

The hazards of government data

Science thrives in a culture of accessibility and transparency that government often finds disadvantageous. Controversies in BSE research and environmental protection illustrate the dangers of secrecy.

WHENEVER scientific research lies close to the sharp end of political debate, the issue of who has access to data takes on a critical importance. If it is governments that control this access, the potential conflict over who best represents the 'public interest' is clear. In such circumstances, researchers in government laboratories are central players, and can therefore be torn, being required to meet the interest of their employers on the one hand and of the wider research community on the other. Current debates in the United States and Europe illustrate how the second of these roles can be all too easily undermined.

Any government agency that collects information with even a potential bearing on policy has a basic responsibility to ensure that such information is preserved for future reference. That statement would have seemed unnecessary were it not for the remarkable action taken last April by the California Environmental Protection Agency (CEPA), instructing scientists in its Office of Environmental Health Hazard Assessment to "dispose of all documents... and other communications prepared during the course of policy formulation which contain other policy proposals not adopted or reflected in the final decision". Disingenuously claiming that its policy was intended to encourage scientists to provide advice without fear of their views becoming known, the agency has now, in the light of protests and court challenges, been ordered by the state government to reverse the instruction (see page 470).

Yet, while almost all government agencies would accept that the need to preserve their stocks of information is self-evident, another seemingly innocuous responsibility can present them with greater difficulty: maintaining such information in a form that is accessible and comprehensible to anybody who may have a subsequent interest in it. Government departments can be abolished and responsibilities transferred, and orderly documentation of files may get disrupted or even destroyed in the process. Such appears to have happened in the United Kingdom, where the abolition of the Milk Marketing Board has made it more difficult for data on dairy herd demography to be obtained, thus obstructing studies of bovine spongiform encephalopathy (BSE).

Even less acceptable is the situation in which sheer lack of resources appears to leave bodies unable to cope fully with their responsibilities. That is said by researchers to be a severe problem at the UK Central Veterinary Laboratory, which plays a critical role in the provision of BSE data — a problem denied by its owner, the Ministry of Agriculture, Fisheries and Food (MAFF) (see page 467).

Keeping documents stored and filed is essentially a duty of care. A further role of government agencies — that of making data available to others — can be more contentious. The CEPA, for example, is now indefensibly seeking to keep its stock of information, saved from destruction, under lock and key. In Europe, a duty of accessibility is made explicit in the case of environmental regulatory bodies that, according to a European directive, are obliged to make available to the public any data that they have collected. But, in practice, activists have found that the lack of a clear definition of which bodies are covered, and the expense of pursuing judicial reviews of refusals of

access, can allow some agencies to wriggle out of their responsibilities. Despite such weaknesses, the European directive provides a welcome boost to openness. Regrettably, it does not apply to non-environmental institutions such as MAFF and its laboratories and their continental equivalents.

Even in countries where freedom of information or 'open government' policies are practised, a desire to protect the interests of suppliers of information — such as businesses, farmers or patients — can allow data to be exempted from the requirement that it is made openly available. But such protection of special interests can often collide with pressures for analyses of a controversial issue by researchers who are independent of government.

The political use of science in the BSE saga highlights the need for such independent analysis as much as any other issue. But even nongovernment scientists argue that access to data should be restricted to those sufficiently sophisticated to interpret its significance responsibly. In particular, they worry about how the mass media or even other scientists can be trusted with data that require a sensitive approach to understand all the necessary caveats. Such concern, for example, lies behind restricted access granted in the United Kingdom to databases of suspected cases of the new variant of Creutzfeldt-Jakob disease, as well as to data on BSE infection. The latter data have often been made available only after strenuous efforts to obtain them — in other words, when the political pressure on MAFF has proved irresistible.

The contrast of that approach to the openness involving data on the AIDS epidemic is striking, while its poor consequences for science are all too obvious. Only one group of nongovernment scientists has had the belated opportunity to conduct an analysis of BSE epidemiology in the United Kingdom. As it happens, that paper (R. M. Anderson *et al.* *Nature* **382**, 779–788; 1996) incorporates results from a separate study of data concurrently submitted to *Nature* that has been publicized as demonstrating maternal transmission of BSE. The latter paper has since been withdrawn, and its senior author, John Wilesmith, is reported to have told a meeting last week that it is now impossible to be sure of the extent to which maternal transmission might be occurring, if at all (*The Veterinary Record* **139**, 328–329; 1996). The algorithm of Anderson *et al.* remains unaffected but, pending further analysis, those seeking to draw policy conclusions from it have an even more uncertain basis than before. Undoubtedly, everybody would be better placed if MAFF had been more open with its data in the past — not only with outside researchers, but also with other government departments.

In short, recent events support a belief that government agencies can, all too often, compromise their commitment to open communication — even between scientific experts — in order to preserve their own interests and those of their 'client' groups. Yet where issues of environmental and health protection are concerned, the need, if only in the cause of good science, to balance this with an equally strong need to meet the interests of the broader community suggests that the case for open access has never been more strong — or more urgent. CEPA's secrecy is being publicly opposed. Who will tackle MAFF? □



Nobel goes to T-cell pioneers whose work 'changed face of immunology'

London. The 1996 Nobel prize for physiology or medicine has been awarded to two immunologists for a pioneering discovery, made more than two decades ago, of the way in which the immune system recognizes virus-infected cells.

The award has been made to Peter C. Doherty, adjunct professor of pathology and paediatrics at the University of Tennessee College of Medicine, and Rolf M. Zinkernagel, head of the institute of immunology at the University of Zurich in Switzerland. It comes as belated recognition for research that had an immediate impact on immunology, and has since paved the way for improved understanding of infectious diseases and chronic inflammatory conditions, such as diabetes and multiple sclerosis.

The Nobel prize is the second award to have been won by the scientists in consecutive years for a discovery that was initially published in April 1974 (see *Nature* 248, 701; 1974) and built upon in October of the same year (see *Nature* 251, 547; 1974). They won the 1995 Albert Lasker Medical Research Award for the same work — reinforcing its reputation as a Nobel 'precursor'.

Their peers were quick to congratulate both scientists as worthy winners who should have been recognized by the Nobel academy earlier. "It's about time," says Polly Matzinger, an immunologist at the National Institutes of Health in Maryland. The research, she adds, "changed the face of immunology. [The award] is wonderful".

Hidde Ploegh, professor of biology at the Massachusetts Institute of Technology, says that "there had never been any question about the importance of this landmark discovery", which provided the direction for

without harming healthy cells.

They also knew that the complicated and intricate job of protecting the body from infection was carried out by T- and B-lymphocyte cells. Researchers had determined that antibodies produced by B-lymphocytes could recognize and eliminate microorganisms, such as bacteria. While it was known how T-cells could reject tissue transplants following recognition of molecules known as major histocompatibility antigens, how these cells recognized virus-infected cells was unclear.

Jonathan Howard, a geneticist at the University of Cologne, says that Doherty and Zinkernagel clarified the role of histocompatibility antigens by using real viruses and set the agenda for the next ten years of research. The breakthrough came when they carried out experiments designed to investigate how T-lymphocytes protect mice against infection from a virus able to cause meningitis.

Mice were infected with the virus and developed virus-killing T-lymphocytes. But, unexpectedly, the scientists found that these T-lymphocytes were unable to kill

cells from other strains of virus-infected mice, demonstrating that an individual mouse's T-cells remain ineffective unless they can also recognize the major histocompatibility antigens of the infected cells.

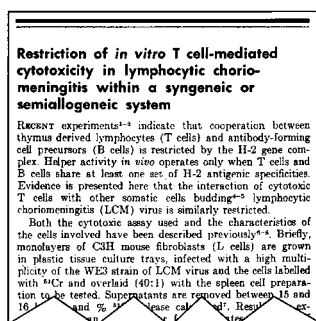
The findings immediately opened up several doors to research into the ability of the body's immune system to recognize and react to microorganisms other than viruses. Further research using a range of molecular tools has since confirmed Doherty and Zinkernagel's original models, and has added important structural detail to their more general picture.

Immunologists now know more about the body's histocompatibility antigens, whose recognition by T-cells is important for eradicating viruses. They have worked out that T-cells recognize a small part of the invading virus — a peptide — that sticks to the body's histocompatibility antigens. Such research has important implications for the development of vaccines.

"This Nobel prize is very appropriate," says Jonathan Sprent, an immunologist at the Scripps Research Institute, La Jolla, California. "So much of immunology is based on what they [Doherty and Zinkernagel] did." **Ehsan Masood & Ursula Weiss**

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Double act: Doherty (left) and Zinkernagel, pictured in 1983, and the 1974 *Nature* paper in which they set out the crucial piece of Nobel-winning research.



future research into the structure and biochemistry of T-cell immunology.

One immunologist, who performed important follow-up experiments, says the prize is "absolutely terrific" news. "I am very lucky to have been totally inspired by their work," says Alain Townsend, professor of immunology at the John Radcliffe Hospital, Oxford, and author of research in the immunology of the influenza virus in the early 1980s. "I used to refer to Rolf [Zinkernagel] as the Bach of immunology."

Doherty was born in 1940 in Australia, where he completed his masters degree. In 1970 he completed a PhD at the University of Edinburgh in Scotland, and joined the John Curtin School of Medical Research in Canberra two years later.

Zinkernagel was born in Switzerland in 1944. He completed his MD thesis at the University of Basel in 1970, and was still a postgraduate research student, not yet having completed his PhD, when he submitted with Doherty the Nobel-winning research to *Nature* in December 1973.

Immunologists at the time knew that lymphocyte cells were capable of destroying foreign, invading, microorganisms as well as the body's own infected cells, but somehow

Rise in US research spending approved

Washington. Spending by the US government on research and development will grow substantially next year for the first time in four years, rising from \$71 billion to \$74 billion. The 4.1 per cent increase is contained in spending bills hurriedly agreed by Congress and President Bill Clinton early last week.

According to an assessment by the American Association for the Advancement of Science, basic research will fare less well than applied research and development, with an overall increase of 2.7 per cent. □

US bioethics panel poised to judge local review boards

Washington. Tighter supervision of the local ethics boards that themselves supervise research protocols is likely to be among the first topics considered by the new National Bioethics Advisory Commission, which is to report within a year on steps needed to protect human research subjects in the United States.

The first task of the 17-member commission, which was appointed in the summer by President Bill Clinton and is chaired by Harold Shapiro, the president of Princeton University, New Jersey, will be to assess the protection of research subjects.

But the commission will also advise both the administration and Congress on other bioethics issues, including the privacy of genetic information, protection of individuals from discrimination based on such data, and the patenting of human genes.

The National Institutes of Health (NIH) and the Department of Energy first asked for the commission to be set up in 1993, to deal with issues raised by genetics research.

Subsequently, however, a separate commission of inquiry into past abuses of the use of human subjects in radiation research found that existing procedures for providing protection were deeply flawed (see *Nature* 377, 374; 1995), and the Clinton administration asked the new commission to focus on this issue first. Its initial two-year mandate expires next October, but is certain to be renewed if Clinton is still in office.

At last week's meeting, several commission members queried the effectiveness of the Institutional Review Boards (IRBs) that approve research protocols at 450 institutions across the United States. Under the rules covering the use of human subjects in publicly funded research, which are voluntarily extended to privately funded research at most institutions receiving public funds, IRBs play a central role in ensuring that subjects are kept safe and properly informed.

Bernard Lo, director of the medical ethics programme at the University of California at San Francisco, said the commission may want "to reconsider if the IRB model [is] really up to the task" of protecting research subjects. Ezekiel Emanuel, another commission member and professor of medicine at the Dana-Farber Cancer Institute in Boston, Massachusetts, said that his experience with IRBs suggested that the system was "too haphazard". These criticisms were echoed by Shapiro, who told a press conference that "at least some of the IRBs do not understand the weight of responsibility that is on their shoulders".

But Gary Ellis, director of the Office of Protection from Research Risks (OPRR) at the NIH, who addressed the commission on

protection of human subjects, defended the IRBs, arguing that they "bring good sense to the table and do the right thing". The main problem, Ellis said, was the lack of legal protection for those who took part in privately funded research not embraced by the current voluntary system. He urged the commission to "articulate a meaningful standard of protection for all human subjects".

Each government agency has submitted a report to the commission on its efforts to protect human subjects, and Shapiro has appointed Jim Childress of the religious studies department at the University of Virginia to head a subpanel to review these. Shapiro promised that the commission will make recommendations on the use of human subjects in research in its first annual report, which is due a year from now.

But the commission has yet to decide which genetics issues it can address. Francis Collins, head of the National Center for Human Genome Research at the NIH, said there was "a critical need to define and enforce conditions for access to and use of genetic information" and that the commission was well-placed to confront the issue.

Collins added that he considered the patenting of human genes a "less promising" area for it to pursue, as the positions of the US Patent and Trademark Office and of the Congress, and the propensity of either to listen to the commission, were unclear.

Indeed, one of the biggest challenges for the commission will be to get its voice heard in the public debate and in the Congress on the issues that it does raise. Administrative questions, such as a reform of IRBs, can be resolved through executive action alone, but larger themes tackled by the commission will require legislation. Whether Congress listens "will depend on the force of our arguments", says Shapiro, adding that Congress is "an important target" for the panel.

The commission will not consider issues that are largely internal to the research community — such as scientific misconduct — or the politically explosive issue of embryo research. A previous National Bioethics Committee collapsed in 1989 after straying into that particular territory.

Jack Gibbons, the president's science adviser, said the commission will draw on work that has been done in other countries, including the United Kingdom. Shapiro said that the group will have an informal meeting with international experts at the forthcoming World Congress on Bioethics in San Francisco at the end of next month. The commission's initial funding level of \$500,000 a year is clearly inadequate, and will be raised to \$1.5–\$2 million, he said.

Colin Macilwain

France unveils plans for 'strategic' gene sequencing centre

Paris. The French government last week announced plans to start the construction of a FFfr1-billion (US\$200-million) gene sequencing centre, with a capacity to sequence 20 to 30 megabases a year. The new centre will be built at Evry, on the outskirts of Paris, already home to the Généthon genome centre.

The centre will be headed by Jean Weissenbach, a researcher at the Centre National de la Recherche Scientifique (CNRS) who works at the Institut Pasteur in Paris. Weissenbach, along with Daniel Cohen, led the pioneering efforts of the Généthon centre to create maps of the entire human genome (see *Nature* 377 Suppl., 367; 1995).

The costs of the centre, which will be set up as a subsidiary of CNRS, are estimated to be FFfr60–FFfr80 million next year, and between FFfr80 and FFfr100 million a year during its planned ten-year lifetime. It will employ 120–140 staff.

François d'Aubert, the secretary of state for research, points out that while France led early efforts to map the genome, it has until now lacked sequencing facilities on the scale of the Sanger Centre in Britain or several laboratories in the United States. The new centre will provide France with an entry into international efforts to sequence the genome, he says.

The announcement of the centre followed the meeting last week of the Interministerial Committee for Scientific and Technological Research (CIRST), a body set up in 1958 by General Charles de Gaulle, but which last met in 1982. The meeting was chaired by Alain Juppé, the prime minister.

The genome centre is one of four 'strategic' programmes announced at the meeting, intended to orient French research more towards the creation of wealth. The others involve support for industrial chemistry, biotechnology and microbiology. The main trade union representing researchers — the SNCS — has already criticized this shift of emphasis as representing a threat to fundamental research, as the money for the new programmes will have to be found from within a shrinking research budget.

One major change that was announced at the meeting, and issued as a decree on the same day, is the abolition of the ceiling on the amount of income that publicly funded researchers may earn from royalties on their inventions. "Researchers can at last become rich from their efforts," said François Bayrou, the minister for education and research.

Declan Butler

BSE researchers bemoan 'ministry secrecy'

Paris. Scientific understanding of the epidemic of bovine spongiform encephalopathy (BSE) in British cattle has been delayed by the reluctance of the UK Ministry of Agriculture, Fisheries and Food (MAFF) to provide access to data, according to UK scientists. This reluctance, they say, has stemmed both from a 'culture of secrecy' at the ministry, and its failure to dedicate sufficient staff to analysing and distributing data on the epidemic.

Direct evidence of secrecy at MAFF comes from the uphill struggle faced by Roy Anderson, professor of zoology at the University of Oxford, and his colleagues, in gaining access to the confidential MAFF statistics needed to produce their recent analysis of the transmission dynamics and epidemiology of the BSE epidemic (see *Nature* **382**, 787; 1996).

Indeed, *Nature* has learnt that MAFF agreed to make the statistics available only after senior officials at the Royal Society put pressure on government ministers, arguing that a credible analysis of MAFF data could be done only by independent experts.

Anderson's study was designed to estimate the number of infected cattle that may have entered the food chain undetected because they were slaughtered before showing clinical symptoms of BSE, and the efficiency of various culling policies designed to reduce the incidence of BSE. Both analyses required raw data on individual farms and the demography of herds, which — according to several sources — MAFF initially refused to provide.

"It was pretty clear that MAFF were scared about the outcome," says one scientist involved in the lengthy negotiations, suggesting that this was because the data would suggest — as they did — that many more sick animals had entered the food chain than was previously thought. MAFF eventually backed down and released the data, he says, after it had been persuaded that an independent epidemiological analysis of the BSE analysis was needed, given that Britain's European partners would be sceptical if this were done by MAFF, which would be perceived as having a vested interest in the outcome — only in this way would other European countries be convinced that the study had been carried out "scientifically and properly".

Anderson declines to comment on this account of events. And a ministry spokeswoman dismisses allegations that it has been overly secretive, claiming that access to data is permitted within agreed collaborative projects. Requests for data that are readily available are met automatically, she says. Those for data that are more difficult to compile must be made through a more formal written procedure that includes a fee calculated on the basis of the amount and

type of data required, and a *pro rata* charge for the salary costs needed to prepare it.

The spokeswoman last week promised that scientists can obtain all the data they need "as long as we deem it possible, and as long as the data doesn't infringe on the confidentiality of individual farmers or the data protection act". She declined to provide a copy of the detailed procedures for obtaining data, however, on the grounds that they had to be formally applied for through so-called 'open government' channels.

Not everyone has had problems. Several researchers acknowledge that MAFF has been helpful in providing relatively small sets of data. They point out that aggregated information has been made available, while MAFF's Central Veterinary Laboratory (CVL) in Weybridge has regularly published epidemiological studies. Similarly, Heino Diringer, a researcher at the Robert Koch Institute in Berlin, says that while he has never asked MAFF for statistical data, he has not encountered problems in obtaining other information.

But one scientist who has had difficulty obtaining data from MAFF complains of a "culture of confidentiality" among government ministries, and MAFF in particular. "This has worked to the detriment of the understanding [of the BSE epidemic] and dissemination of information in general," he says, pointing out that the health and environment ministries have better track records "in terms of being open and involving scientists from outside government".

"It has been a nightmare to get hold of comprehensive data," says John Kent, a statistician at the University of Leeds, adding that "not having the numbers more easily available has made life difficult". Kent is keen that MAFF should now make widely available the data used in the Anderson study so that they can be critically assessed.

Anderson agrees that this is necessary. But he points out that the database is not his to give, and that scientists must request it from MAFF. Mark Savey, a leading French epidemiologist working on BSE, says he has been given assurances by MAFF scientists that he will be given access to the particular datasets he wants.

Allegations of excessive secrecy within MAFF are confirmed by one MAFF scientist involved in BSE research. He says that while wider access to data on BSE might not have had much practical impact on the handling of the epidemic, MAFF's lack of openness has been "deplorable". "There is a general principle of not wanting to give the data to anybody. But then the political pressure became so great that it had to be given to the Anderson group," he says. "We shouldn't have been able to withhold data."

The MAFF spokeswoman defends the ministry's actions, however, arguing that its BSE database contains confidential information on individual farms, whose release is forbidden by current government policy; such databases must also respect the provisions of data protection legislation.

Anderson agrees that these factors are important. But he says that they are not insurmountable obstacles to the release of data, pointing out that the databases relating to the AIDS epidemic were made available to the entire scientific community "for analysis and interpretation".

Similarly, the spokeswoman argues that the general release of data could be misleading in the wrong hands. This view is supported by several scientists. "You can't make primary data widely available because it's too complicated," says one. "You need to first make a synthesis." Uncontrolled release of data could cause more problems than it solves, he says, through misinterpretation by both scientists and the press. ▶

Hubble reveals shadow of Jupiter's moon

This unusual picture of Jupiter and its volcanic moon Io shows the moon's shadow, about 3,600 km in diameter, sweeping across the planet's cloud cover at a speed of 17 km a second.

Taken in July at violet wavelengths by the Hubble Space Telescope, using its Wide Field Planetary Camera 2, the image, released last week, is one of a series intended to complement those being taken by the Galileo spacecraft now orbiting Jupiter. □

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J. Spencer (Lowell Observatory) and NASA

► There is general agreement that complex databases should not simply be made freely available, as their proper exploitation requires an understanding of how the data have been assembled, and the various caveats they contain. But many scientists say that analogous problems in other areas — such as census data — are overcome by employing individuals to provide such assistance. The CVL would need an extra dozen people to do this, says one MAFF scientist. "It would take a lot of resources, but I think it would be well worth doing."

Indeed, MAFF's failure to assign sufficient staff to the analysis and distribution of BSE data appears to have been a major factor contributing to the difficulties faced by outside scientists. Graham Medley, a biostatistician at the University of Warwick, says he has benefited from greater cooperation from MAFF after having complained on a television programme about the prob-



lems of gaining access to MAFF data. Since then he has encountered few such difficulties, and attributes any that have arisen simply to the fact that the CVL is "inundated" with requests, and is "grossly understaffed".

Statistical research on the BSE epidemic within MAFF has been left mainly to a handful of researchers at the CVL. The small numbers of people involved is "staggering for a problem of this importance", says one scientist. Anderson says that, given the scale of the BSE problem, MAFF should have quickly assembled "a large and effective team collaborating with external teams with expertise in particular areas", and that this should have included researchers from other European countries.

Moreover, Anderson argues that cutbacks in government science over the past decade have made such external input more important than ever. These cuts have reduced the government's capacity to carry out the in-house research required to deal with complex scientific issues in many areas, he says. What is now needed, argues Anderson, is an interministerial body — perhaps organized by the Office of Science and Technology — that would assess important scientific issues, decide whether outside expertise was needed, and, if so, arrange for it to be brought in quickly. **Declan Butler**

New Zealand scientists seek to revive political fortunes

Sydney. Spending on research by the government of New Zealand has recently begun a slow upturn after a lengthy period of decline. But the scientific and academic communities are still seeking the support of the political parties taking part in the general election on 12 October for further improvements to funding, especially for universities.

Scientists continue to express concern at the effects on research and university teaching of the cost-cutting and restructuring that accompanied the rapid shift towards 'user pays' and 'privatization' or 'corporatization' of public services over the past few years. These policies were introduced by a Labour government and extended by their National Party successors.

But the scientists' campaign is clouded by a new and unpredictable electoral system. Twenty-seven parties are vying for 120 seats under a 'mixed-member proportional' system that has replaced the traditional first-past-the-post method of electing 99 members to the single-chamber parliament.

The New Zealand Association of Scientists (NZAS) and the Royal Society of New Zealand have lobbied for public funding to be restored to the levels that applied when the cuts began 15 years ago. New Zealand spent 1.03 per cent of its gross domestic product on research and development (R&D) in 1993, placing it fourth from the bottom out of 24 leading economies. Private-sector spending on R&D is even lower on this list.

Since 1989, responsibility for policy advice to government, funding decisions and the provision of research has been allocated to separate bodies. And scientific merit has been replaced by relevance to national needs as the main criterion for selecting projects to be supported. In 1992, as part of this trend, Simon Upton, the Minister of Research, Science and Technology, abolished the government's Department of Scientific and Industrial Research, replacing it with ten sector-based and commercially oriented Crown Research Institutes (CRIs).

The government justified these changes on the grounds that research performance needed to be more focused in a small country. Claiming last year that the changes have been a success, Upton produced a plan for the next 15 years, which aims to lift government expenditure to 0.8 per cent of gross domestic product.

In particular, Upton has reversed the downward trend of government funding through the new Marsden Fund (NZ\$25 million by next year) for merit-based 'blue skies' research outside the government's

priority-setting process and promised that the Public Good Science Fund, which supports projects in CRIs and universities, will be increased and its short-term grants extended.

But many scientists have been disillusioned by the impact of a 30 per cent decline in government funding since 1981. The NZAS has described this decline as "disastrous". A survey of the 300 members of the association in the academic community, government and industry found that the scientific workforce had been "traumatized and decimated" and its productivity "greatly reduced".

According to three past and present officials of the association, writing in the September issue of *NZ Science Review*, the CRIs have had "varying success, with some functioning well, but others experiencing difficulties, and one small CRI collapsed". Even the effect of the Marsden Fund in sustaining basic knowledge is uncertain, they write.

Despite recent improvements, the survey concludes that morale among scientists is low. "Particularly those in the CRIs were unhappy about their management, which was viewed to be hierarchical, authoritarian, short-term focused and secretive." Job security, it says, has been lost, salaries have declined, time spent on research has decreased, international regard has fallen, and freedom to publish and speak out on policy issues has been restricted.

The governing, conservative National Party has dropped sharply in the opinion polls to 35 per cent. In a recent lecture, Upton promised to boost environmental research through a 'green package' and called for a pause in "tinkering" further with science. Labour, led by Helen Clark, is closing on National with a popular promise to reduce student fees, but is vague about whether it will increase public spending on R&D, claiming it will be interventionist and will not leave investment to the market.

The left-wing Alliance, which promises to abolish student fees altogether, is polling level with NZ First, a party which has now been overtaken by Labour. Its leader, Winston Peters, a populist Maori defector from the Nationals, proposes to "increase public investment in science and technology to the median level of New Zealand's major trading partners" — although without saying where the money will come from.

In higher education, universities, polytechnics and associations of staff and students have joined in a Public Tertiary Education Coalition to oppose the funding cuts planned for the next three years by the National Party. **Peter Pockley**

NASA pulls plug on comet collaboration

Washington. The United States has pulled out as a major contributor in the European Rosetta mission to visit a comet early in the next century. The move has angered European space scientists, and left participants on both sides dismayed at the sudden breakdown in an international partnership that had been going well.

Wesley Huntress, head of science at the US National Aeronautics and Space Administration (NASA), told Roger Bonnet, his counterpart at the European Space Agency (ESA), at a meeting last month in Washington, that NASA's "funding profile" for the next several years will not allow the agency to deliver the comet lander vehicle it was planning to supply on the schedule that ESA had requested.

Although Rosetta is due to launch in 2003, ESA had asked for the lander — which will separate from the main orbiter spacecraft on arrival at the comet — to be delivered 18 months early. The spacecraft was originally to have carried two landers, one built by Germany (called RoLand), and the other built by NASA and the French space agency CNES, called Champollion.

Several months ago, however, ESA decided that weight and budget restrictions for Rosetta, one of the agency's 'cornerstone' science missions, would allow only one lander. That left German, US and French engineers scrambling to combine their two designs into one.

NASA and some US investigators on the Champollion project say that engineering problems alone would make such a merger difficult, if not impossible. The two vehicles had different anchoring systems, and RoLand was designed for a longer stay on the comet's surface.

But others, such as Roger Yelle of Boston University, one of the US investigators on Champollion, believe that issues of politics and ownership have played at least as large a role as the engineering problems. "If the decision-makers in this process wanted to [cooperate], they could do it," he says.

Jean-Pierre Bibring, of the Institut d'Astrophysique Spatiale (IAS) in Paris,

who was the CNES co-project scientist for Champollion, sees NASA's withdrawal as ultimately due to a lack of US interest in keeping the collaboration going. "They did their best not to merge the two [designs] into one," he says.

Some US scientists, however, say that ESA must shoulder part of the blame for the breakdown, having made the original decision to cut back to one lander. ESA also was unable to accommodate NASA's request to deliver Champollion later, they say. Some on the US side point out that NASA is mak-

US investment in Champollion will still be applied to some kind of comet mission, as NASA continues to regard this as a high scientific priority. Despite many aborted plans in the past 15 years, the United States has yet to launch a spacecraft to study a comet. CNES will be invited to participate in any US mission, says Rahe.

If NASA does fly its own comet mission, what had been a successful international collaboration will turn into a competition. The US spacecraft would launch at around the same time as Rosetta in 2003, but would use advanced solar electric propulsion to reach a still-to-be-determined cometary target in only three or four years — well ahead of Rosetta's arrival at Comet Wirtanen in 2011.

NASA/CNES

One problem with the New Millennium option, however, is that this programme is designed primarily to test new spacecraft technologies. Some US scientists fear that research would therefore take a back seat, whereas Champollion was clearly a science-driven mission.

JPL's report on the New Millennium option is due next month,

along with decisions on who will be involved in the remaining Rosetta lander.

Officially, ESA and NASA say only that they "regret" the situation, and that this will have no bearing on their relationship. But the affair is likely to put a chill on future space science collaborations — similar to, if not as severe as, the ill-will caused by the US withdrawal from full partnership in the International Solar Polar (now Ulysses) project in the 1980s.

The agencies are already telling different stories about what happened. NASA paints its withdrawal from Champollion as a joint decision between the Europeans and Americans. That has angered some Europeans, who say that CNES was not fully consulted before NASA decided to pull out of the project at the September meeting in Washington. Giacomo Cavallo, Bonnet's deputy in ESA's science directorate, says that while it was a "friendly meeting", it also was "clear it was a NASA decision".

Rahe believes cooperation on the Rosetta mission can still go smoothly. The three US investigators flying experiments on the main orbiting spacecraft should not be affected by the Champollion decision. But Rahe — as well as other space scientists — admits that collaboration on spacecraft projects in the era of "better, cheaper, faster" is harder than it used to be.

With more volatile budgets and less flexibility in their schedules, space agencies on both sides of the Atlantic increasingly face the need to change their plans quickly, which makes international consensus much more difficult to achieve. **Tony Reichhardt**

IMAGE
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Champollion may merge with the German RoLand project.

ing no such demand for early delivery of the European Huygens probe due to fly on the US Cassini mission to Saturn next year.

With the United States out, representatives from ESA, the RoLand project and CNES met last week to assess options for a merged European lander.

Germany, which has been having its own financial difficulties in keeping RoLand afloat, has invited NASA to participate in this reassessment, and engineers at the Jet Propulsion Laboratory (JPL) in Pasadena, California, are still exploring opportunities for US involvement. But few are optimistic that this will come to pass.

NASA, meanwhile, is considering other plans. A study group at JPL is looking at the possibility of flying the cometary science instruments that were to have flown on Champollion on another US lander vehicle, perhaps as a technology test flight under the agency's 'New Millennium' programme.

Jurgen Rahe, NASA's head of planetary exploration, says the planned \$120-million

Germany lends Israel helping hand in Brussels

Munich. Israeli scientists are to benefit from help and advice provided by the German liaison office for the European Commission (KOWI) in their applications to the Commission for research grants under the Fourth Framework programme (FP4). Israel signed an agreement with the European Union at the beginning of August which gives its scientists the right to participate, at their own cost, in all of the framework programmes.

Last month, KOWI agreed with Israel's seven universities that it would offer their scientists the same service that it offers to German scientists in helping them to obtain funding from Brussels.

In line with this commitment, KOWI will help Israeli applicants to find their way around the various programmes within FP4, and help them find partners in other European countries, an essential prerequisite for participation. □

California backs off destruction of records

San Francisco. California's state Environmental Protection Agency (Cal-EPA) has suspended a controversial plan to destroy records of dissenting scientific opinions about risk to public health from industrial emissions, chemicals and hazardous waste.

Under a 'records retention policy' introduced last spring, the agency ordered state scientists to destroy research data and internal records that differed from final policy directives on hazard assessment.

The policy would affect documents maintained by the Office of Environmental Health Hazard Assessment, which makes policy on toxicity, carcinogenicity and safety measures related to agricultural chemicals, hazardous wastes and industrial emissions.

Cal-EPA said the policy would foster free exchange of opinion by protecting the identity and views of the scientists involved. But the move was criticized by scientists, environmentalist groups and free-speech advocates, who claim that the policy was designed to do just the opposite — namely to censor debate about public health risks.

Last week, a few hours after a public-

interest lawsuit charged the agency with violating the public's right to know about the workings of government, Pete Wilson, the governor of the state, ordered the agency to maintain its records and clarify any confusion over the policy. Cal-EPA agreed not to destroy the documents, but said it would keep them in confidential files.

The Natural Resources Defense Council, the Environmental Law Center and the Society of Professional Journalists had filed a joint suit against the agency in San Francisco. They said Cal-EPA had no legal basis to keep the documents secret, and was violating state public-records laws.

Cal-EPA has the right to keep some documents confidential. But according to Terry Francke, director of the California First Amendment Coalition, a free-speech watchdog group, such action requires the status of the documents to be resolved in court. Destroying documents, he says, subverts California's open-records laws by making such court scrutiny impossible.

Francke argues that the agency's action conflicts with both public access to

government operations and the norms of scientific practice. "The presupposition in the development of science is that all information should not only be public, but widely disseminated so discussion can advance on the validity of the research and its findings," he says.

Other protests have come from the California Association of Professional Scientists, a labour union for scientists employed by the state. They argue that scientific integrity would be compromised under the new policy, and that important data on public health would be destroyed.

A memorandum ordering the enactment of the plan instructed state employees to dispose of all documents and other communications prepared during the course of policy formulation "which contain other policy proposals not adopted or reflected in the final decision". Correspondence, electronic messages and scientific data would all be assessed on whether the information they contained was reflected in policy. If in doubt, the documentation was to be destroyed.

Sally Lehrman

Companies cool to tactics of global warming lobby

London. The Global Climate Coalition (GCC), the US energy lobby group that has challenged some of the main conclusions of United Nations climate scientists about the severity of manmade global warming, has suffered a setback to its lobbying efforts with the resignation of two member companies and the possibility of further withdrawals.

BP America, a subsidiary of British Petroleum, and the Arizona Public Service Company, a Phoenix-based electric power utility, are pulling out of the group, which earlier this year accused scientists working for the Intergovernmental Panel on Climate Change (IPCC) of distorting the conclusions of a key report in order to over-emphasize the role of human activities in climate change (see *Nature* 383, 287; 1996).

Klaus Kohlhase, head environment adviser at BP headquarters in London, says that BP felt its interests in the United States were not best represented by remaining in the coalition. But BP, he says, will stay a member of the International Climate Change Partnership, an alternative industry lobby group which he describes as "a more moderate and conciliatory" body.

Mark De Michele, chief executive of the Arizona Public Service Company, partly shares that sentiment. "Global climate change is a serious problem and we need to take steps to deal with it," says De Michele, one of the architects of a Clinton administration-backed initiative to persuade power utilities voluntarily to reduce greenhouse gas emissions. "I was concerned that to continue

to attack the science — which the GCC is basically doing — is not the way forward."

Representatives of environmentalist groups, such as the US Climate Action Network, the Natural Resources Defense Council and Greenpeace, say that the resignations are a significant development. Paul Horsman, head of oil campaigns at Greenpeace, claims that some members of the energy industry are realizing that the GCC has lost credibility and are re-grouping around other organizations, such as the climate change partnership and the Business Council for a Sustainable Energy Future.

But Bill O'Keefe, president of GCC and a vice-president of the American Petroleum Institute, shrugs off the resignations. "We're adding members, not losing them," he says. "We have eight to ten new board members, and are still the leading business voice in climate change."

Both BP and the Arizona Public Service Company, says GCC's executive director John Shlaes, were 'general' members, a category he describes as "an educational forum". 'Board' membership, on the other hand, entitles companies to influence GCC policy, and costs US\$20,000 annually (general members pay US\$2,500).

This summer, the GCC mounted a vociferous campaign to contest a report written by scientists working for the IPCC, which had been re-edited just before publication. The GCC claimed that the alterations amounted to "scientific cleansing". The scientists said the changes were necessary to

clarify parts of the text that had earlier confused policy-makers. The ensuing war of words caught the attention of Republican members of the US Senate, who convened a series of hearings into the matter.

Several GCC member companies are understood to have been uneasy about the organization's aggressive tactics. And in July, a senior US administration official weighed in by indirectly referring to the GCC as "naysayers and special interests bent on belittling, attacking and obfuscating climate change science", in a speech to the annual conference of the climate convention in Geneva (see *Nature* 382, 287; 1996).

But the withdrawal of the Arizona Public Service Company is also believed to have been influenced from another quarter — the Interfaith Center on Corporate Responsibility, an association of institutional investors representing US churches and other religious-based investments. The centre, which claims to represent combined investments of US\$70 billion, has asked each GCC member company — where its affiliates are shareholders — to resign from the coalition.

Ariane van Buren, director of the centre's environment programme, says the GCC should not be allowed to do further harm to the process of dealing with global climate change. "In no case should [a member] company allow its name to be associated with any additional GCC activities that distort the facts." Companies, she says, should join "responsible business coalitions".

Ehsan Masood

Russian documents set out 'tectonic weapon' research

Moscow. The first official details have emerged in Moscow of ambitious research into 'tectonic warfare' carried out by the former Soviet Union and subsequently by the government of Russia, and involving attempts to stimulate 'artificial' earthquakes as weapons of destruction.

According to documents obtained by the newspaper *Moscow News*, two research programmes, the first known as 'Mercury' and the second as 'Volcano', were aimed at creating new earthquake epicentres by using underground nuclear explosions.

Geophysicists are aware that impending earthquakes may be triggered by underground nuclear explosions. But Western geophysicists remain sceptical about tectonic warfare and have all but abandoned research after two unsuccessful phases of activity in the 1960s and 1980s, says Roger Clark, a lecturer in geophysics at the University of Leeds.

Clark is not at all surprised that the Russians tried to create earthquakes and control their location electromagnetically, however. "This sort of science is very much part of their heritage. We don't think it is impossible, or wrong, but past experience suggests it is very, very unlikely."

The programme, which was secretly launched by the Communist rulers of the former Soviet Union in 1987, and has been unofficially known to Western geophysicists for several years, is now believed to have been abandoned. It would certainly contravene the terms of the Comprehensive Test Ban Treaty, which Russia signed at the United Nations in Geneva last month.

The Mercury project was launched in the former Soviet republic of Azerbaijan, but came to a halt when the republic became independent. It was superseded by the Volcano project. Three underground nuclear tests are believed to have taken place at sites in Kyrgyzstan.

According to the documents, the Mercury project was launched by a secret decree of the Central Committee of the Communist Party and the Council of Ministers of the Soviet Union. The objective was to "develop a methodology for remote operation on an earthquake epicentre by using weak seismic fields and research possibilities of transferring the seismic energy of an explosion".

The documents say that the Mercury project involved 22 scientific and industrial organizations, including the Geological Institute of the Azerbaijan Academy of Sciences in Baku. The remit extended to developing the electronic equipment to be installed aboard space satellites that would control the tectonic weapon. The scientists were given three years to complete research,

with testing planned for 1990.

During the research phase, Azerbaijani scientists grew increasingly confident and, according to the documents, were sure that "after [a] nuclear explosion, subterranean energy may accumulate at huge distances from the epicentre and reach massive capacity, after which the next directed explosion can release it all".

Underground testing began at the town of Batken in Kyrgyzstan, and was directed by Ikram Kerimov, of the Azerbaijan Academy of Sciences. The documents say that scientists detonated an underground nuclear charge and tried to control the direction of seismic energy released using British-built equipment known as 'system 9690'.

A report prepared by the Mozhaisky Military Engineering Institute concluded that the test had been a success. But progress slowed considerably following Azerbaijan's independence from the Soviet Union. At about this time, Russia embarked on a more comprehensive tectonic warfare programme known as the Volcano project. The Earth Physics Institute of the Russian Academy of Sciences (RAS) became the project headquarters.

Research was scheduled to be completed in 1992, with underground testing beginning the following year. The final test was carried out at a place code-named S36NZ-0Kh; *Moscow News* believes the letters 'NZ' refer to Novaya Zemlya, where Soviet nuclear testing began in the 1950s. **Carl Levitin**

Precautionary route for risk management backed by Chirac

Paris. Jacques Chirac, the French president, said last week endorsed the "precautionary principle" in the management of risks associated with science and technology, as well as the promotion of ethical debate as a way of reconciling society with the changes brought about by such advances.

Chirac, speaking at the opening of the fourth annual meeting of Unesco's International Bioethics Committee in Paris, said

that debates about ethics should not be seen as a "rejection of modernity". Rather, they are a "means of founding modern citizenship" by encouraging discussion about scientific progress and public understanding of the issues it raises.

But he added that ethical debate does not remove decision-making responsibility from politicians, saying that the role of scientific experts is to give impartial advice, not to make decisions themselves. Referring to the 'mad cow affair', Chirac said he endorsed the precautionary principle in decision-making — that politicians should "not hesitate to favour the worst-case scenario" — whenever the potential consequences could be disastrous. **Declan Butler**

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Chirac: ethical debate furthers understanding.

Italy joins call for more coordination

Munich. The research programmes of the European Union (EU) should be more closely coordinated with the work of national research organizations, according to the Italian government. The proposal comes in Italy's position paper on the European Commission's fifth Framework research programme (FP5), which is due to begin in 1998.

The paper reflects the influence of Antonio Ruberti, a former EU research commissioner, who heavily promoted the concept of cooperation and coordination with member states during his term of office in 1993–94. He is now head of the Italian parliament's committee on science and technology.

According to the paper, the commission should invoke articles in the Maastricht Treaty that allow it to support complementary research outside the main Framework programme that involves a limited number of member

states. Such arrangements, known as 'variable geometry', also have the support of Germany and the Netherlands (see *Nature* **381**, 634; 1996). They could either involve a 'top-down' initiative from the commission, or a 'bottom-up' initiative of two or more member states or national research organizations.

FP5 should concentrate on three main themes, the paper says: quality of life and environment, the information society, and sustainable production. It lists possible objectives within these areas, including development of vaccines, elucidation of the neurological basis of diseases of ageing, and future energy options, including nuclear fusion.

The Italian paper also includes a plea for the planned international fusion reactor, called ITER, to be sited in Europe — even though no European country has expressed a wish to host the reactor. **Alison Abbott**

Pressure groups seek labelling of genetically engineered foods

Washington. EuroCommerce, an umbrella group representing a number of European food wholesalers and retailers, this week demanded the labelling of exports of a genetically engineered soybean produced by the US company Monsanto Co., and due to reach the world market this month, to indicate they have been genetically altered. Simultaneously, Jeremy Rifkin, president of the pressure group Foundation on Economic Trends, announced a worldwide boycott of foods not guaranteed free of Monsanto's soybeans, and of a genetically engineered corn produced in the United States by CIBA-Geigy Corporation. The ten targeted foods include Coca Cola and McDonald's french fries. Rifkin says that the engineered plants pose unacceptable environmental and health risks.

The governments of the United States, Canada, Japan, Mexico, Argentina and the European Union (EU) have approved the soybeans as safe, although in the EU approval is restricted to import and processing. The United States, Canada and Japan have approved the corn. The EU failed earlier this year to approve it, referring the issue to several standing scientific committees. □

Gulf veterans' health review

Washington. The Pentagon has asked the National Academy of Sciences and the Institute of Medicine for yet another study into its handling of health problems afflicting Gulf War veterans. John White, the deputy defence secretary, said last week that the new study will look at how the Department of Defense (DoD) could improve plans to help troops cope with "unfamiliar environments".

Meanwhile a separate panel of the Institute of Medicine has called on the DoD to establish a uniform electronic medical record sys-

tem for Gulf War veterans. The panel's report, which was requested by Congress, says that the approach of research by the DoD and the Department of Veterans' Affairs into the problem had improved over the last two years, but that their capability to do medical and population-based research still needs further strengthening. □

'Ig Nobel' research highlighted

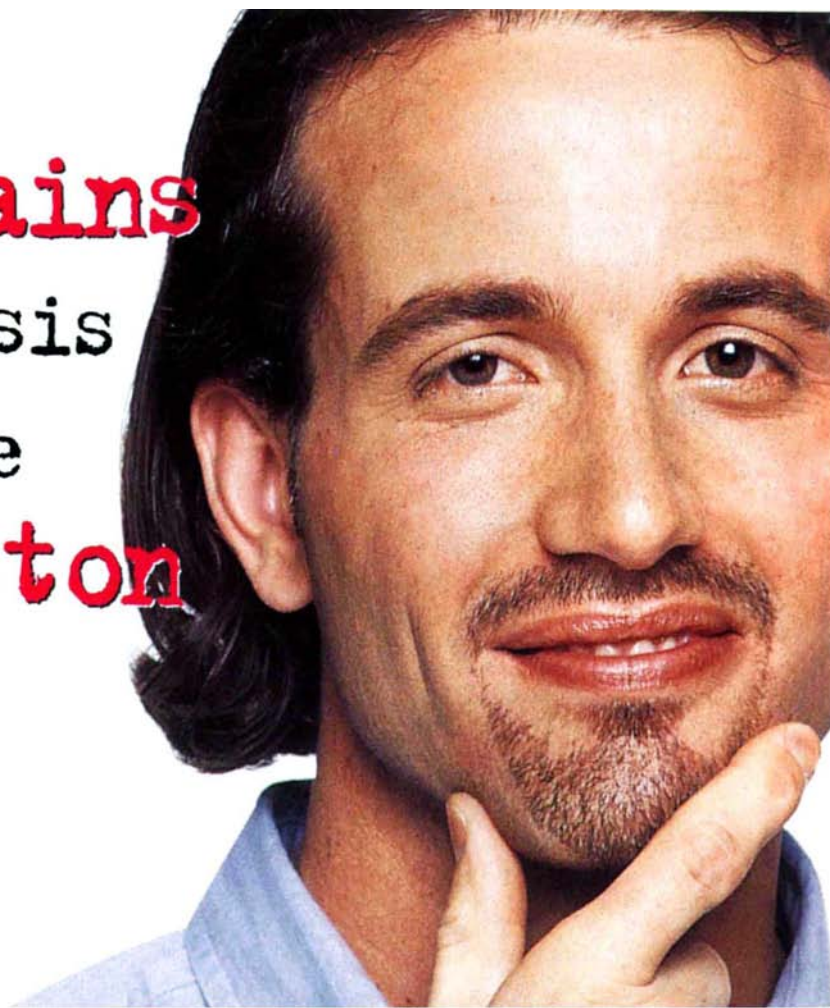
Boston. Three tobacco company scientists last week shared the spoof 'Ig Nobel' prize for medicine for discovering that "nicotine is not addictive". The biology prize, awarded during a ceremony at Harvard University, went to a group of Norwegian researchers for a study of the effect of "ale, garlic and soured cream" on the appetite of leeches, and the physics prize to a British scientist for a paper on why dropped toast always lands "butter-side down". The awards, which attracted criticism from Britain's chief scientist for ridiculing serious scientific effort (see *Nature* 383, 291; 1996), are made to mark achievements that "cannot or should not be reproduced". □

Official arrested in HIV scandal

Tokyo. Akihito Matsumura, a former official of the Ministry of Health and Welfare, last week became the first government official to be arrested in Japan's HIV-contaminated blood scandal. This follows a series of arrests, including those of the president and two former presidents of Green Cross Corporation, a leading Japanese blood-product manufacturer (see *Nature* 383, 291; 1996) and Takeshi Abe, one of the scientists at the centre of the scandal that led to the infection of almost half of Japan's 5,000 haemophiliacs with HIV through the use of non-heat-treated blood products in the 1980s.

Matsumura was in charge of the ministry's Biologics and Antibiotics Division between July 1984 and June 1986, and was arrested on suspicion of professional negligence resulting in death, for failing to order the withdrawal of non-heat-treated blood products despite

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allegedly knowing about the risks of HIV infection in November 1984. He resigned from the ministry in July this year, when investigations began. Charges of murder have also been filed against him in the Tokyo and Osaka Courts by the relatives of two victims. □

Marine industry grants warning

London. The Engineering and Physical Sciences Research Council (EPSRC) is in the unusual position of being attacked for reducing its spending on industry-related research in order to spend more on grant applications from the academic community. The complaint has come from the Marine Technology Directorate Ltd (MTD), a body set up 20 years ago to support joint government-industry projects in oil and gas research, and ship design.

MTD warned last week that proposals from the EPSRC's advisory panels that the council should withdraw from targeted research in marine technology and concentrate on 'responsive mode' funding, "threaten to destroy the concept of collaborative research partnerships, based on industry needs". Richard Brook, chief executive of the EPSRC, says it plans to continue supporting research under the marine technology programme, but acknowledges that this is one area in which cuts are under consideration. □

Russian scientists strike

Moscow. Nationwide strikes have been announced by the labour unions representing Russian scientists, triggered by a growing financial crisis in research institutes attached to the Russian Academy of Sciences. Scientists working for the academy have not received salaries for three months, and many are threatening to join colleagues in a hunger strike to back their claims. The financial situation has been made worse by the fact that institutes belonging to the academy have received only 15 per cent of the government funds they were due to receive for the three months of July to September. □

Au revoir to anthropology?

Paris. The worst fears of researchers at the Musée de l'Homme (Museum of Mankind) in Paris were realized this week when Jacques Chirac, the French president, endorsed a proposal to turn the museum into a centre of 'primitive art' that was put forward last month by a commission he had set up (see *Nature* 383, 203; 1996). The new centre will be known as the 'Museum of Civilizations and Primitive Arts'.

The decision appears to doom an alternative proposal by the Natural History Museum, the museum's parent body, to renovate the existing museum while preserving its commitment to a scientifically-based exhibition of its collections. Chirac's rapid decision to adopt the commission's recommendations will fuel the complaints of some researchers that the commission was simply a front to endorse a *fait accompli*. The Natural History Museum held a press conference on Monday, just before the release of Chirac's decision, in a last-minute bid to influence the proposal, which it says it will continue to fight. □

Support for 'green taxes'

London. A report released last week by the European Environment Agency (EEA) in Copenhagen says that 'green taxes' imposed deliberately to achieve environmental goals appear to be successful in meeting their objective, and should be used more often. Particularly successful applications of such taxes include those imposed on sulphur dioxide and nitrogen oxides in Sweden, on toxic waste in Baden-Württemberg in Germany, and on water pollution in the Netherlands. While admitting that serious political barriers exist to the introduction of such taxes, it says that evidence indicates that these can be overcome by "careful design and extensive consultation". But it admits that the overall impact of such taxes remains relatively small — representing less than 1.5 per cent of total taxation in 1993 — and is increasing only slowly. □

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Individuals' rights and wrongs

SIR — By chance, I happen to be well acquainted with the three 'case studies' presented by your correspondent Rex Dalton (*Nature* **383**, 107; 1996). I interviewed Timothy Baldwin for a research position in this department a couple of years ago; I was a member of an appointments committee for another post for which Kimon Angelides applied (but was not appointed); and I have known Annmarie Surprenant for many years.

I should like to make two points:

(1) In the case of Baldwin and Angelides, no hint of any possible misbehaviour was contained in the letters of reference received from their previous employers in the United States. It was only through indirect (verbal) contacts with others that I learnt of some of the matters referred to in Dalton's article. I can understand that employers may be chary of committing doubts in writing where no certain evidence exists, but I think the onus is on them to communicate such doubts in some form, perhaps by telephone or e-mail.

(2) The case of Surprenant is quite different: whatever misdemeanour she may have committed in the legal sense, there is no evidence whatsoever that this translates to her scientific research, which has been substantial, significant and reproducible.

Nature should take care to distinguish between scientific misconduct or fraud in the sense of fraudulent or misleading science and social or legal misconduct that happens to be committed by scientists: the two are not the same, and the latter may have no bearing on the veracity of their work.

David Brown

Department of Pharmacology,
University College London,
Gower Street,
London WC1E 6BT, UK

SIR — With the publication of the News story "International recruitment highlights need to track scientific behaviour", *Nature* has lowered itself to the level of tabloid journalism. While this may be good for circulation, it does irreparable damage to science and may be harmful to both the health and reputation of individual scientists.

The basic premise underlying this article is incorrect. How many examples are there of scientists who have moved to Europe after inquiries into their activities in the United States? Furthermore, how many of these assumed positions where their new employer was actually unaware of the former difficulties of the scientist in question? In at least two of the three cases cited, the employers knew of the previous difficulties these scientists had encountered, and the scientists are now carrying out their teach-

ing and research duties at their new institutions admirably. Why then rehash previous difficulties that they may have had, especially if these issues have still not been completely resolved?

You should understand the personal consequences of the type of character assassination reported in the article. Even if these scientists had made mistakes, is it not conceivable that they have learned from them and are now capable of making substantive contributions to teaching and research?

Jeffrey Rosen

Department of Cell Biology,
Baylor College of Medicine,
1 Baylor Plaza,
Houston, Texas 77030-3498, USA

Blake's heaven

SIR — I thought it rather ironic that the editors of *Nature* chose to place William Blake's painting of Newton on the cover of the reprint of the human genetic linkage map article (*Nature* **380**, 152–154; 1996). Blake was a mystic, poet and anti-rationalist who had no tolerance for the ideas arising from the European Enlightenment, upon which the philosophical and scientific foundations of this current work are based. Further, this specific painting reflects Blake's view that Newton, looking down to the ground, absorbed with his protractor and paper, was completely oblivious to the magnificence and splendour within and around him.

Regardless of the intention of the editors, I suspect that Blake's followers would wholly approve of the cover choice — as if a DNA sequence could possibly describe the totality of the human experience.

David L. Bain

Department of Medicine,
University of Colorado
Health Sciences Center,
4200 E 9th Ave, Box B-151,
Denver, Colorado 80262, USA

Smoke signals

SIR — I would like to clarify three points raised by Flint¹ and Britton² in response to my Commentary article on the media handling of preliminary scientific data³.

First, I am the genetic toxicologist who is so well known to Oliver Flint and me. I remain grateful for the sample of thalidomide that he gave me in 1984. The extensive negative mutagenicity data that my colleagues and I have subsequently generated on this chemical have enabled us to classify it with confidence as a nonmutagen, despite the earlier publication of positive muta-

genicity data. A preliminary discussion of our data enabled us to counter recent media scare stories regarding the possible germ-cell mutagenicity of this agent to humans⁴.

Second, my comments on butyl benzyl phthalate were neutral and concerned only the way in which preliminary biological data on this chemical were being discussed by the media.

Finally, I evidently trapped myself in the snare of irony when I referred to the assertion that the hazard of passive smoking could be equated to the eating of three hamburgers a day. My use of the word "curiously" was intended to convey my rejection of the analogy drawn. Its bankrupt nature is realized when one attempts to calculate how many hamburgers a day a heavy smoker is implied to have eaten, or when one imagines the consequences to the health of a baby of its being force-fed three hamburgers each day. My views on the advertisement in question are exactly those of Britton².

John Ashby

Zeneca Central
Toxicology Laboratory,
Alderley Park, Cheshire SK10 4TJ, UK

1. Flint, O. *Nature* **382**, 572 (1996).
2. Britton, J. *Nature* **382**, 572 (1996).
3. Ashby, J. *Nature* **382**, 109 (1996).
4. Ashby, J. & Tinwell, H. *Nature* **375**, 453 (1995).

Cause and defect

SIR — You recently published a review of my book *The Economic Laws of Scientific Research* (*Nature* **382**, 123–124; 1996). More recently, David Swinbanks reported on Singapore's research funding (*Nature* **383**, 110; 1996), and his report seems to illustrate those laws beautifully.

Between 1978 and 1991, Singapore's expenditure on research and development (R&D) rose as its gross domestic product (GDP) per capita rose (the first law). In 1991, however, the government set up the National Science and Technology Board with a budget of S\$2 billion, and what happened? After 1992, the rate of growth in the total R&D budget fell, and that budget has since failed to rise above 1.1% of GDP. This disappointment is compatible with the displacement of private funding by the government's money (the second law), a displacement that is disproportionate and damaging to the overall R&D budget (the third law).

The economic laws of scientific research are not an academic conceit. They show how the government funding of research harms the enterprise.

Terence Kealey

University of Cambridge,
Department of Clinical Biochemistry,
Box 232, Addenbrooke's Hospital,
Cambridge CB2 2QR, UK

Phytoplankton bloom on iron rations

Bruce W. Frost

An iron-enrichment experiment in the equatorial Pacific Ocean shows that increased availability of this trace nutrient induces dramatic biological and biogeochemical changes in the surface waters.

EXPERIMENTS in the open ocean are particularly challenging. Take the study of biological and biogeochemical processes occurring near the ocean surface. An unambiguous test of some hypotheses about these processes practically dictates an *in situ* experiment, but lateral and vertical exchanges due to advection and turbulent diffusion impose severe logistical difficulties on the approach. Fortunately, the technology for such experiments now exists. It has been successfully applied to a salient oceanographic question, as described in a remarkable study by Coale *et al.* (page 495 of this issue¹) and companion papers by Behrenfeld *et al.*², Cooper *et al.*³ and Turner *et al.*⁴ which begin on page 508.

The question is, what regulates phytoplankton abundance and production in the equatorial upwelling zone of the eastern Pacific Ocean? The major nutrients required for phytoplankton growth, such as nitrate and phosphate, are available there in high concentrations, yet phytoplankton seem unable to use them efficiently, and phytoplankton abundance remains low. These features are common to the three so-called 'high-nitrate, low-chlorophyll' (HNLC) regions, which include, besides the eastern equatorial Pacific, the ice-free Southern Ocean and the open subarctic North Pacific Ocean. Clarifying the regulatory mechanisms concerned is necessary for understanding ocean ecology, the oceanic carbon cycle, and the involvement of the upper-ocean biota in transfer of carbon from the atmosphere to the ocean depths.

The papers report an experimental test of the late John H. Martin's hypothesis⁵ that it is the availability of iron that limits phytoplankton in the HNLC regions. Iron is nearly insoluble in sea water, but is an essential trace nutrient required by phytoplankton for many biochemical processes (chlorophyll synthesis and nitrate reduction, for example). Measurements showing that surface-water concentrations of dissolved iron are extraordinarily low (sub-nanomolar) led Martin to first test the iron hypothesis using shipboard nutrient bioassays. Natural phytoplankton incubated in bottles usually increased in abundance when provided with extra iron. But bottles of sea water as microcosms of the ocean are always suspect. So an *in situ* iron-fertilization experiment was designed

and has been carried out twice, first in 1993 and then again last year.

In the first experiment (IronEx I), reported without fanfare in this journal two years ago⁶⁻⁸, a single dose of iron, raising the dissolved iron concentration to 4 nM in a 64-km² patch, resulted in significant increases in phytoplankton abundance and production rate, but had little effect on nitrate concentration or partial pressure of CO₂. Sadly, the experiment ended prematurely when the fertilized patch sank beneath a layer of less dense water; the ecosystem response to iron became attenuated for unknown reasons. Although the experiment verified the iron hypothesis, it left the issue of whether iron fertilization could relieve the HNLC condition unresolved^{9,10}.

In contrast, during the second experiment (IronEx II, described in this issue), the fertilized patch remained at the surface and retained its integrity while drifting 1,500 km. The same amount of iron was added as in IronEx I, but in three sequential infusions over a week to effect a more sustained increase of dissolved iron in the surface layer of a 72-km² patch. The fertilization had an immediate and dramatic effect. Within the enriched

patch, phytoplankton photosynthetic capacity, growth rate and abundance increased, and nitrate decreased. As the phytoplankton bloomed, its species composition changed radically. Diatoms became dominant and accounted for most of the increased use of nitrate¹. Significantly, these events parallel those observed in previous shipboard nutrient bioassays in the equatorial Pacific¹¹.

The fate of the added iron is not reported, but the stimulated biological production was short-lived. Within a week of the last iron infusion, indicators of phytoplankton physiological condition returned to the same levels as those in the iron-limited ambient waters² and the phytoplankton bloom waned, presumably due to grazing and sinking (although the tracer budget indicated mixing losses as well)³. Contrary to expectation¹², there was no sign that iron was retained and efficiently recycled within the biota of the surface layer. So episodic natural inputs of iron, either from the atmosphere⁵ or from below¹³, should induce episodic pulses of short duration in phytoplankton abundance and production. The consequences of larger-scale, longer-term inputs of iron are uncertain; from observations in the

IronEx II — the principal results

In IronEx II, dissolved iron was infused from a ship into the surface layer of a square patch of ocean in the eastern equatorial Pacific. The measurable growth response of phytoplankton to nutrient enrichment occurs on a time-scale of days, so the success of the experiment hinged on staying with the fertilized patch as it drifted 10–100 km per day in the current. This was accomplished by tagging the patch with a drogued buoy and a biologically inert tracer (SF₆) added with the iron. The response of the natural, unenclosed plankton assemblage to iron was monitored by repeated surveys of the patch over several days. Untreated water outside the tagged patch served as a control. The main results are these:

- Specific growth rate (cell division rate) of the phytoplankton doubled, phytoplankton abundance increased

by more than 20 times (approaching levels observed in coastal phytoplankton blooms), and nitrate concentration declined by half. Larger sizes of phytoplankton, chiefly diatoms, dominated¹.

- Indicators of phytoplankton photosynthetic capacity (photochemical energy conversion efficiency, light absorption capability) showed immediate and sustained increases².

- As the bloom developed, the partial pressure of CO₂ in the centre of the patch decreased rapidly, reducing the ocean-to-atmosphere CO₂ flux by 60 per cent³.

- The concentration of dimethyl sulphide (DMS), a potential biogenic precursor of atmospheric sulphate particles which may be implicated in modifying climate through their effects on global albedo, increased by more than three times in the bloom⁴.

iron-replete surface waters of the equatorial Atlantic upwelling region, however, it seems that a new ecosystem state would ensue, with higher phytoplankton production and depletion of major nutrients, but relatively low phytoplankton stocks, perhaps regulated by more abundant grazers.

What did IronEx II reveal about the usual workings of the pelagic ecosystem in the eastern equatorial Pacific? Availability of iron unequivocally limits cell division rates, abundance and production rate in phytoplankton. In retrospect, the earlier nutrient bioassays in bottles showed this. Nevertheless, the *in situ* experiment was necessary to test the possibility that a rapid response of grazing zooplankton could counter the effect of iron fertilization by cropping the phytoplankton¹⁰. But although small and large zooplankton increased in abundance and grazing rate¹, the response was insufficient to prevent the diatom bloom.

IronEx II also reinforces the notion that, together with grazing, iron limitation determines the composition of natural assemblages of phytoplankton¹¹. Although the phytoplankton as a group is limited in division rate by iron availability, different types seem to be affected to different degrees. The dominant, very small species normally responsible for most of the community photosynthesis have moderate, or even high¹⁴, division rates that appear to be roughly balanced by losses from grazing¹. Iron fertilization did comparatively little to disrupt this grazing balance; even though the photosynthetic capacity of small cells increased greatly, their abundance only doubled in five days. This was to be expected, as the main grazers of small phytoplankton in the eastern equatorial Pacific are protozoans¹⁵ that can potentially grow as fast or faster than phytoplankton.

But what of the larger-sized phyto-

plankton, particularly the diatoms that became dominant under iron fertilization? These species are probably under severest iron stress¹⁶ in HNLC regions, and ought to have low division rates, although this is yet to be demonstrated in the equatorial Pacific and there is some contradictory evidence¹⁵. To fully understand the dynamics of iron enrichment, natural and experimental, the *in situ* division and mortality rates of large diatoms need to be determined, and we need to know more about the major grazers on diatoms and how they respond when diatom abundance increases.

Underlying debate about the iron hypothesis is the possibility that iron-enhanced phytoplankton production might influence levels of atmospheric CO₂ by increasing the flux of carbon into the surface ocean and on into the ocean depths in sinking particles⁵. The induced phytoplankton bloom in IronEx II significantly reduced CO₂ concentrations in the

surface layer³. Yet models suggest that even complete alleviation of iron limitation in the HNLC regions would have only a small effect on future global atmospheric CO₂ (refs 17, 18). The past may be something else, however, as there is evidence of higher, possibly iron-induced, phytoplankton production in parts of the Southern Ocean during the last glacial stage¹⁹, when atmospheric CO₂ was lower. An additional wrinkle from IronEx II is the observed increase, in the bloom, of dimethyl sulphide (DMS)³, which may have a role in global climate. Phytoplankton species that produce the precursor of DMS are particularly abundant in Antarctic waters. So one of the next steps is to find out how the regulatory mechanisms revealed in IronEx II apply to the Southern Ocean pelagic ecosystem. □

Bruce W. Frost is in the School of Oceanography, University of Washington, Seattle, Washington 98195-7940, USA.

ALZHEIMER'S DISEASE

Tangle disentanglement

Konrad Beyreuther and Colin L. Masters

In Alzheimer's disease (AD), intellectual decline is associated with two types of fibrous-protein deposition in the brain — abundant extracellular fibrils of β -amyloid, and intracellular fibrils of polymerized tau. In consequence, researchers have become divided into two camps with different views as to the primary cause of the disease. One is the β -amyloid protein party of the 'Baptists'; the other is the so-called 'tauists'. The discovery¹ of mutations in three genes which segregate with AD in families, and appear to influence β -amyloid biogenesis, has lent strong support to the Baptists. This case cannot yet be made for tau, leaving its proponents in something of a dilemma.

A report in this issue may, however, point to a way out. Goedert *et al.*² (page 550) provide compelling evidence that the assembly of tau into fibrils requires the presence of an additional factor, glycosaminoglycans (GAGs), which have been shown to be associated with both amyloid plaques and the neurofibrillary tangles composed of tau³.

In AD brain, the intracellular tau lesions are found in nerve cell bodies and apical dendrites as neurofibrillary tangles, in remote dendrites as neurofibrillary threads, and in the abnormal nerve endings (neurites) associated with β -amyloid plaques. These latter, extracellular deposits are initially nonfibrillar but are progressively transformed into fibrils, and they consist mainly of the 42-amino-acid β A4 peptide (β A4/42), a proteolytic product of the amyloid precursor protein (APP)⁴. Nerve

cells with neurofibrillary tangles and synapses within the region covered by amyloid plaques are believed to undergo degeneration, become dysfunctional and result in AD.

Each tangle contains paired helical filaments (PHFs) as major components and single straight filaments (SSFs) as minor components. All six isoforms of the microtubule-associated protein tau are components of these filaments⁵ (see figure). Because the degree of tau phosphorylation is the only known difference between PHF-tau and normal microtubule-associated tau, and because PHF-tau in its hyperphosphorylated state does not bind to microtubules, it had been believed that abnormal phosphorylation of tau is a necessary prerequisite for its self-assembly into PHFs and SSFs⁵.

After neuronal death, PHF-tau may survive owing to its relative insolubility, and accumulate in the neuropil as ghost tangles. Part of the amino and carboxyl termini of tau are cleaved in these extracellular tangles, implying that the core of the PHF is made up of the three or four tandem repeat regions of tau which normally function as a microtubule-binding domain (ref. 5; see figure). Only the shortened, three-repeat tau fragments have been shown to assemble into paired helical-like fragments *in vitro*⁶. All attempts to form PHFs or SSFs from recombinant full-length tau, either unphosphorylated or phosphorylated, gave unsatisfying results⁷. This suggested that the self-assembly of bacterially expressed whole

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tau used for these studies may require phosphorylation at specific sites which had not been achieved *in vitro*, or that additional factors were needed.

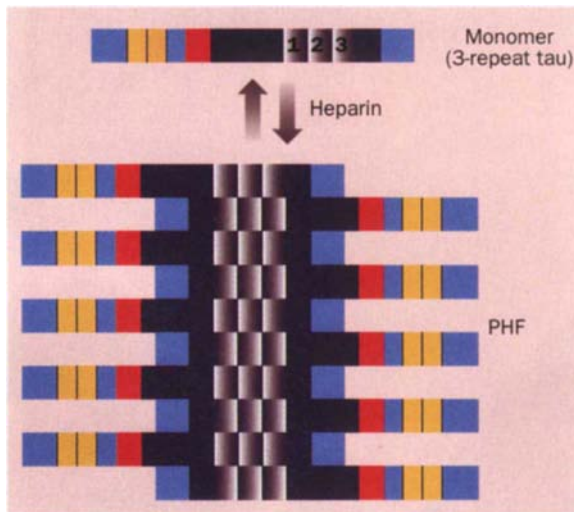
Goedert *et al.*² now show that addition of GAGs such as heparin, heparan sulphate, chondroitin sulphate or dermatan sulphate is the key (see figure). They report that assembly of PHFs results from three-repeat tau and of SSFs from four-repeat tau. Independently, similar results have also been found for fragments of tau encompassing the microtubule-binding repeat region⁸. Phosphorylation of tau is not required, and does not inhibit or promote PHF formation. These observations on PHF and SSF production indicate that post-translational modifications are not essential for tau assembly *in vitro*, and raise several questions.

What, for example, is the cause of PHF formation in AD, where the process occurs in the presence of all six tau isoforms? The existence of hyperphosphorylated and *N*-glycosylated tau in PHF has led to the proposition that abnormal phosphorylation of tau disrupts microtubules, and causes tau assembly into straight filaments which are then crosslinked into PHF (ref. 9). Goedert *et al.* clearly show that heparin also inhibits tau binding to microtubules and leads to microtubule disassembly. They demonstrate that heparan sulphate and phosphorylated tau coexist in some nerve cells of an AD brain, extending previous studies which showed that GAGs stimulate tau phosphorylation¹⁰. They also provide some evidence that accumulation of neuronal heparan sulphate may precede tangle formation.

Other issues concern the mechanism by which amyloid plaques form, their role in the disease process and the relationship between tangles and plaques. The discovery of autosomal dominant mutations in APP and presenilins 1 and 2, which segregate with AD, poses the questions of whether these mutations primarily affect the amyloidogenic metabolism of APP and amyloid formation, or cause disease in other ways^{1,11}.

The demonstration that not only APP but also presenilin mutations result in

increased production of β A4/42 (ref. 11), the predominant β A4 subunit of plaques which is believed to be highly prone to amyloid fibril formation¹², indicates that β A4 deposition seems to be the neuropathological process most strongly influenced by these genetic factors. But how could this process produce neuro-



Assembly of paired helical filaments (PHFs) from tau; PHFs are a major component of the neurofibrillary tangles characteristic of Alzheimer's disease. Six brain tau isoforms are produced from the tau gene. The process involves alternative messenger RNA splicing of two amino-terminal exons (yellow) and of a fourth tandem repeat region, which in four-repeat tau is inserted between repeat 1 and 2 (shown in silver) in the carboxyl-terminal half. These three (marked 1–3) or four tandem repeats constitute the microtubule-binding domain of tau and are believed to stabilize microtubules. Heparin and phosphorylation negatively regulate this binding and stabilization. Tau has several sites for heparin binding in the amino-terminal fragment, comprising the acidic (blue), basic (red) and proline-rich (black) regions, and in the carboxyl-terminal half which includes the repeat region. Goedert *et al.*² demonstrate that, in the presence of heparin, all three recombinant tau isoforms which contain three microtubule-binding repeats, and tau fragments comprising only this repeat, assemble into PHFs in a phosphorylation-independent fashion. This shows that the structural requirement for PHF formation is contained within the tau microtubule-binding repeat region.

degeneration in AD? One possibility is that amyloid filaments interact with cell-surface receptors of neurons and microglia to induce production of neurotoxic free-radicals and cytokines, but the data are somewhat contradictory¹³. A mechanism leading to apoptosis which is independent of β A4 has also been proposed¹⁴.

As to the relationship between the formation of β A4 amyloid plaques and neurofibrillary tangles, it seems that tangles do not cause plaques because the

latter are absent in several other but rarer neurodegenerative diseases that are also characterized by tangles or related neuronal inclusions¹⁵. On the other hand, the process leading to amyloid-fibril formation could cause tangles, because mutations in the APP gene result in increased amounts of β A4/42, amyloid plaques and neurofibrillary-tangle formation^{1,11}.

Amyloid formation might therefore provide a key factor in tau assembly. Goedert *et al.* suggest that GAGs could be this factor. Heparan sulphate GAGs have been immunolocalized to amyloid plaques and neurofibrillary tangles, and chondroitin sulphate GAGs have been immunolocalized within tangles and neurites⁵. Heparan sulphate GAGs bind with high affinity to β A4 peptide and APP (refs 1, 16), which could explain their accumulation in β A4 plaques and neurites enriched in APP. Furthermore, the chondroitin sulphate GAG chain of L-APP isoforms (lacking exon 15)¹ could directly promote tau polymerization if the two molecules were in the same compartment.

Any interaction of tau, a cytoplasmic protein, with GAGs and GAG side-chains of transmembrane proteins such as APP, as seems possible in AD, requires a membrane abnormality; that is because glycosylation in normal cells takes place in the lumen of the rough endoplasmic reticulum and the Golgi apparatus. Such an abnormality could be brought about by β A4- or APP-induced oxidative stress^{13,16} or damage to membranes, and could also explain how altered neuronal cholesterol uptake mediated by apolipoprotein E is associated with increased density of β A4 amyloid deposition in both late-onset familial and sporadic AD (ref. 11).

Studies of the processes involved in the interaction of the cytoplasmic protein tau with luminal GAGs promise to provide clues to the mechanisms linking amyloid filament formation with neurofibrillary degeneration in AD.

As an immediate outcome, however, the discovery of Goedert *et al.* provides tauists with an assay for the identification of compounds that prevent PHF formation. So they are now in a position that the baptists achieved some time ago¹². □

Konrad Beyreuther is in the Center for Molecular Biology (ZMBH), University of Heidelberg, Im Neuenheimer Feld 282, D-69120 Heidelberg, Germany (e-mail: beyreuther@mail.zmbh.uni-heidelberg.de). Colin L. Masters is in the Department of Pathology, University of Melbourne, Parkville, Victoria 3052, Australia (e-mail: c.masters@pathology.unimelb.edu.au).

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Just out of pigtails

Mitchell A. Berger

How, in a simple and forceful way, do we characterize the dynamics of systems with several moving components? When the components move in two dimensions, methods based on the theory of braids may provide the answer. That is why an experiment on the motion of beads drifting in a magnetized fluid¹ will be of general interest to those studying nonlinear dynamics. The space-time diagrams reveal a rich topological structure that would not be readily apparent in a motion picture of the beads.

People generally visualize objects as they exist at one instant of time; in a drawing, a one-dimensional curve represents the position and shape of a filament. But just over a century ago in *The Time Machine*, H. G. Wells advocated a different way of looking at objects — as they exist in both space and time. This adds an extra dimension: in a diagram with one axis representing the time coordinate, particles generate one-dimensional curves, and a loop of string becomes a tube. A decade later, special relativity made this viewpoint fundamental to our understanding of nature. Even though most scientific work does not involve relativistic effects, time generally does play a role, and researchers may well wonder how their data would look as plotted in a space-time diagram.

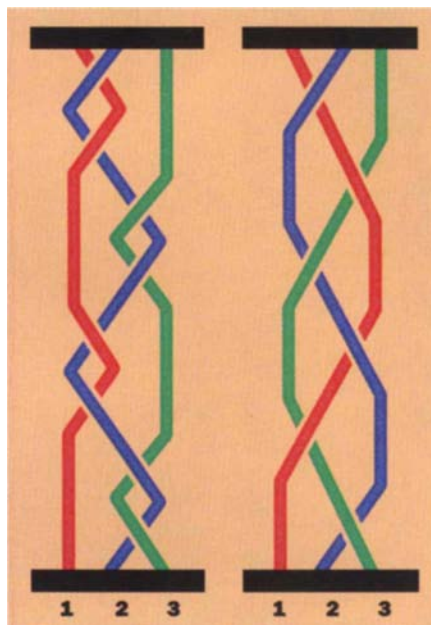
In the new experiment by Pieranski *et al.*¹, non-magnetic spherical beads are placed in a thin layer of fluid containing iron particles. A rotating magnetic field sets the fluid in motion. The beads behave like magnetic holes, and interact with one another according to simple, but nonlinear, equations. The essentially two-dimensional motion of a bead can be represented as a curve in a three-dimensional space-time diagram, and so several beads in motion produce a set of braided curves.

The authors suggest that the topological description of this braid provides a simple and concise language for describing the dynamics of the system. Here one ignores all the little wiggles in the motions of the magnetic holes, concentrating instead on the overall pattern of movement. The holes perform a complicated dance as they move about one another, and the braid encodes the choreography of this dance.

To understand what is meant by the topology of a braid, think of a set of strings stretching between two parallel planes (see figure), crossing one another at several places. Emil Artin in 1925 gave a simple way of keeping track of the crossings, and hence describing the braid structure. Just below each crossing, label the strings 1, 2, 3... from left to right. If string 2 crosses over string 3, label the crossing

σ_2 ; if it crosses under string 3, label the crossing σ_2^{-1} (the character σ is just something to attach subscripts and superscripts to). The entire braid can be coded as a sequence of these symbols.

Suppose we fix the ends of the strings at the two boundary planes, but deform the strings in between. Such an operation is said to preserve the topology of the braid,



Two braid diagrams. Although their sequences of crossings are different ($\sigma_2^{-1}\sigma_2^{-1}\sigma_1^{-1}\sigma_1^{-1}\sigma_2\sigma_2\sigma_1\sigma_1$ and $\sigma_2^{-1}\sigma_1\sigma_2^{-1}\sigma_1\sigma_2^{-1}\sigma_1$; see text), they are topologically equivalent. The right-hand braid is the standard pattern for plaiting pigtails.

even though the sequence of Artin symbols may change. For example, a braid with the sequence $\sigma_2\sigma_1\sigma_2$ can readily be converted to one with the sequence $\sigma_1\sigma_2\sigma_1$. Simple algebraic algorithms can be used to check whether two sequences belong to the same braid topology².

Once a sequence of motions has been converted into braid notation, it can be further examined to obtain numbers characterizing the structure and complexity of the braid. For example, the number of positive crossings minus the number of negative crossings (those with the -1 superscript) is a topological invariant known as the 'writhe', which characterizes the net twist of the braid. And the minimum possible length of the braid sequence provides a measure of complexity. At present, simple algorithms exist only for minimizing braids with three strings³.

The algebra of braids has applications in several areas of mathematics and theoretical physics, including statistical mechanics and field theory⁴. In 1983, Joan

Birman and R. F. Williams developed mathematical tools for analysing the knottedness of trajectories⁵. Since then, braid theory, a subset of knot theory, has been a particularly rich source of insight⁶. For example, Alan McRobie and Mike Thompson used braid diagrams to look at the trajectories of nonlinear oscillators⁷.

To illustrate this technique, consider a ship rolling in heavy seas. On the x - y plane, plot the angle of roll against the velocity of rolling, and then follow the evolution of these two coordinates with time. A set of a few different initial values will lead to trajectories that trace out a braid, and the braid topology will change as the parameters of the physical system change — dramatically so when there is a bifurcation or when qualitatively new behaviour emerges. In this case, "qualitatively new behaviour" can mean the ship capsizing.

Braid theory has applications in astrophysics as well⁸. X-ray pictures of the Sun often show clouds of gas as long loops or arches; these loops trace out the direction of the magnetic lines of force, and often display a complex braided and twisted structure that reflects the pattern of motion of magnetic flux at the surface of the Sun. Braid complexity correlates with magnetic energy storage, and violent changes in the braid pattern can release this energy, heating the solar atmosphere to millions of degrees and accelerating charged particles to high energy. We may be able to predict such magnetic storms by monitoring the complexity of the braids.

Theoretical work by Christopher Moore⁹ and the experiment by Pieranski *et al.* point to new directions for research. Moore looked at two-dimensional dynamical systems in general, and proved that any braid type can be realized as a set of trajectories in some dynamical system. But linking braid structures to particular systems, especially those that occur in natural or experimental settings, remains to be done.

Braids generated by random mechanisms can also be usefully studied. The tangle of wires under your desk or behind your stereo system may prove to be a ripe source of investigation. □

Mitchell A. Berger is in the Department of Mathematics, University College London, London WC1E 6BT, UK.

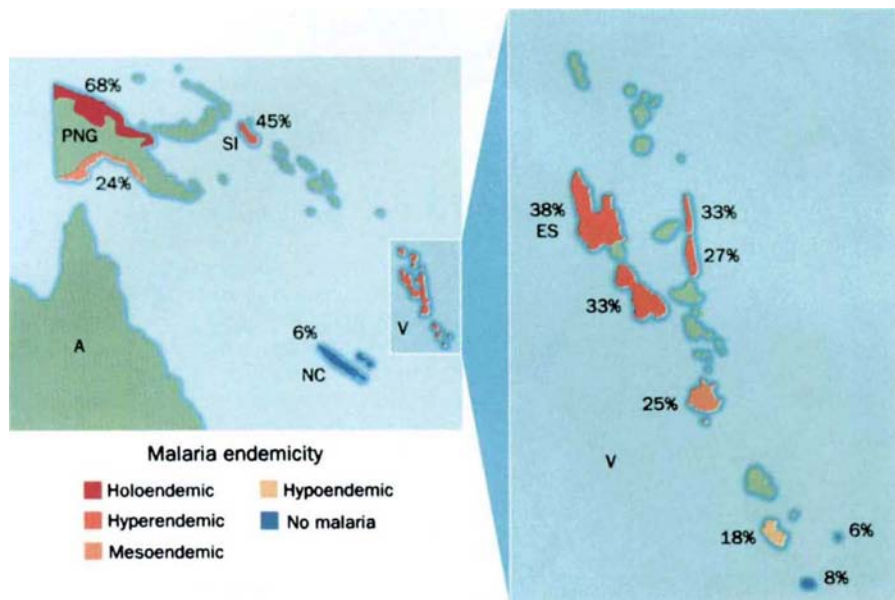
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Protective selective pressure

Louis H. Miller

THE high mortality from *Plasmodium falciparum* malaria^{1,2} has drastically modified the human genome by the selection of genetic variants that reduce the risk of dying from malaria. One such genetic abnormality, presumed to be protective, is alpha (α^+)-thalassaemia, produced by the deletion of one of the two duplicated α -globin genes that encode this component

other red-cell defects, such as sickle haemoglobin, β -thalassaemia and glucose-6-phosphate dehydrogenase deficiency. These genetic disorders protect against severe life-threatening malaria and, in case-control studies⁵⁻⁷ and one prospective study⁸, they are also associated with protection from non-severe or mild malaria. Williams *et al.* propose that



The percentage gene frequencies (numbers on map) of α^+ -thalassaemia in Melanesia parallels the intensity of malaria transmission (endemicity). The malaria transmission intensity goes from most intense (holoendemic) to least intense (hypoendemic). A, Australia; PNG, Papua New Guinea; SI, Solomon Islands; NC, New Caledonia; V, Vanuatu; ES, Espiritu Santo — an island in Vanuatu where the study by Williams *et al.* was performed. Figure adapted from the data in Flint *et al.*³.

of haemoglobin and which are found on each chromosome. This assumption has been based on the general global correlation of α^+ -thalassaemia frequencies with malaria endemicity and on a detailed study in Melanesia, where the gene frequency of 68 per cent in an area of intense malaria transmission falls to less than 10 per cent in areas of no transmission³ (see figure).

Williams *et al.*⁴ (page 522 of this issue) tested the malaria-protection hypothesis by determining the incidence of malaria attacks (fever from malaria) in children on the island of Espiritu Santo in Vanuatu, expecting that the children who had α^+ -thalassaemia would have fewer attacks of malaria. Instead, they found that there was an increased incidence of malaria in children who were homozygous for α^+ -thalassaemia, when compared with those who were heterozygous or who had no gene deletions. Paradoxically, the authors interpret this as being evidence for a protective effect of α^+ -thalassaemia against dying from malaria.

The finding of Williams *et al.* seems to be at odds with findings from studies of

the protective mechanism of α^+ -thalassaemia against malaria may result from an increased incidence of mild malaria in early childhood, which generates increased immunity and reduces the likelihood of a subsequent life-threatening severe attack — this is a new proposal.

How might this increase in the number of malaria attacks in childhood lead to protection against dying from malaria? The answer is complicated by the presence of two malarial parasites on Espiritu Santo — *P. falciparum*, and the less virulent *P. vivax*, both of which cause more malaria attacks in children with homozygous α^+ -thalassaemia. One interpretation assumes that this genotype is associated with more mild, and fewer severe, attacks of *P. falciparum* malaria, even in the absence of *P. vivax*. Williams *et al.* propose that this effect might result from more parasitic invasion of the younger red blood cells found in thalassaemics⁹. In this way, frequent episodes of mild malaria may generate immunity against more severe malaria.

The authors suggest that the observed high expression of malarial antigens on α^+ -

thalassaemic red cells¹⁰ might facilitate the rapid development of protective immunity, but it is unclear why this would not then also protect against mild malaria. For the beneficial effect of increased immunogenicity to outweigh the deleterious effect of increased parasite growth, most of the increased susceptibility to malaria should occur during the period of relative malaria resistance in early infancy. But more data are required to test this fascinating scenario of evolutionary brinkmanship, with the host sacrificing health for survival.

A second interpretation of the data invokes a role for the other parasite, *P. vivax*. This species has been far less frequently studied by human geneticists, and little is known about how it may be affected by abnormalities in haemoglobin. Williams *et al.* speculate that the increased number of clinical attacks of *P. vivax* in children with α^+ -thalassaemia may lead to protection from subsequent fatal infections with *P. falciparum*. But this speculation, and the assumed avirulence of *P. vivax*, may not be correct. Infection with *P. vivax* has been used in the past as a therapy for neurosyphilis, but it was found that this did not reduce the severity of subsequent *P. falciparum* infections¹¹ (although a possible disease-modifying interaction in the field remains to be studied). Also, the near absence of the Duffy blood-group antigen (the Duffy-negative phenotype) on red cells in West Africans suggests that complete resistance to *P. vivax* has a selective advantage. *Plasmodium vivax* requires the Duffy blood-group antigen as a receptor for invasion, so Africans with the Duffy-negative phenotype cannot be infected by *P. vivax*¹², although *P. falciparum* is unaffected. Could *P. vivax* itself, within the context of other diseases, cause mortality and select for α^+ -thalassaemia? In 1929, S. P. James presented evidence that *P. vivax*, which was thought to be the predominant cause of malaria in Britain 100 years ago, was then the cause of much severe disease and death¹³.

Malaria-resistance genes provide some of the best illustrations of natural selec-

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tion in humans, and have provided important insights into pathogenesis and possible strategies for vaccine and drug development. Analysis of immunogenetic associations with malaria has recently encouraged the development of cytotoxic T-lymphocyte-inducing vaccines against the liver stage of the malaria parasite¹⁴, and has supported intervention studies targeting tumour necrosis factor in cerebral malaria¹⁵. Whatever the mechanism of protection by α^+ -thalassaemia, the unexpected result of Williams *et al.* calls into question some of the assumptions regarding the natural history of the development of severe disease.

We have probably identified only a minority of the genes that influence resistance to malaria. Where the infection is a major cause of mortality, many genetic

polymorphisms may be selected. What is needed is a broader approach to evaluate the entire human genome for factors that influence susceptibility or resistance to malaria. Methods for screening the genome for disease susceptibility and resistance genes in general are now well established. To apply them to malaria would require a huge investment in recruiting hundreds of multi-case families in areas where severe malaria is prevalent. But, judging by the lessons that this parasite and its resistance genes have taught us so far, that should be worth the effort. □

Louis H. Miller is at the Laboratory of Parasitic Diseases, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Maryland 20892, USA.

OPTICAL MATERIALS

New stack system for records

Nasser Peyghambarian and Bernard Kippelen

FOR storing huge quantities of information, optical holographic recording is an attractive alternative to the more conventional magnetic technology. The main factor limiting large-scale manufacture of such systems is the lack of a low-cost and easily processed recording medium. With the development of photorefractive crystals, this technology gained some momentum, and, more recently, polymeric materials such as photorefractive polymers^{1,2} have emerged that can be easily processed into large-area films at low cost. On page 505 of this issue³, Berg and colleagues add a new, supramolecular candidate to the list of potential recording media for holographic storage: peptide oligomers.

In holographic storage, an image is recorded in an optically sensitive material as an interference pattern, formed by a laser beam bearing the image and a second reference beam (Fig. 1). The image can be retrieved by diffracting a reading beam off

the pattern recorded in the medium. Because all the information is moving in and out in parallel, the access rate is high. Also, by using different wavelengths, polarizations and reference-beam angles, many images can be independently stored in the same volume of material, allowing storage densities of hundreds of gigabits cm^{-3} .

The information is usually encoded by light-induced changes in the medium's refractive index. One elegant way to modify the refractive index of a material is to use rod-like molecules as dopants, and control their alignment with a laser beam. That can be done by using the conformational changes of azo dyes: the geometry of the molecule changes when excited by light, until eventually its axis lies perpendicular to the polarization of the light. This effect has been used to store holograms in polymers doped with azo dyes, and the same dyes have been used as molecular rotors to align liquid crystal films⁴.

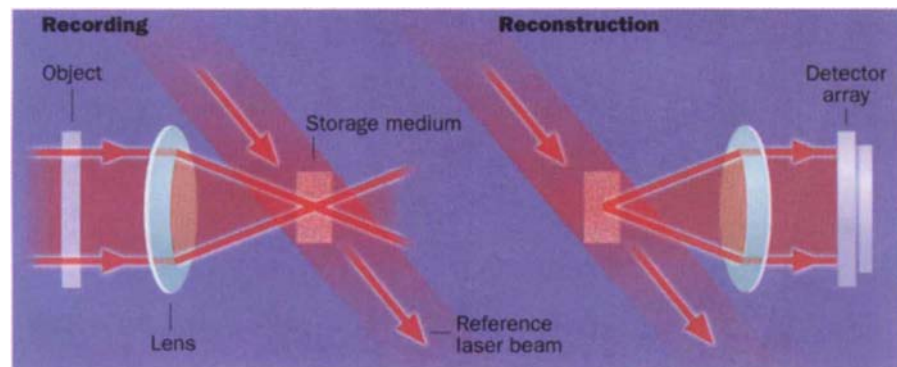


FIG. 1 Two-dimensional arrays of information can be stored as holographic images. Two laser beams are used, one a reference beam, and the other passing through the image to be stored. The beams interfere in the medium, and the pattern formed is optically encoded as variations in refractive index. Information stored in this way can be retrieved by diffracting a laser beam off the pattern, or erased by exposing the medium to a uniform light source.

But Berg *et al.*³ add a new dimension to the tailoring of organic molecules for photonic applications. They show that by using supramolecular architectures from biology, the performance of such materials can be improved. By linking the functional molecules to a peptide-like backbone, they can impose helical stacking in a manner similar to that in DNA. The planar azo-benzene molecules orientate themselves until their planes are in a direction perpendicular to the light field, as they would do singly, but being coordinated by the backbone they cannot rotate

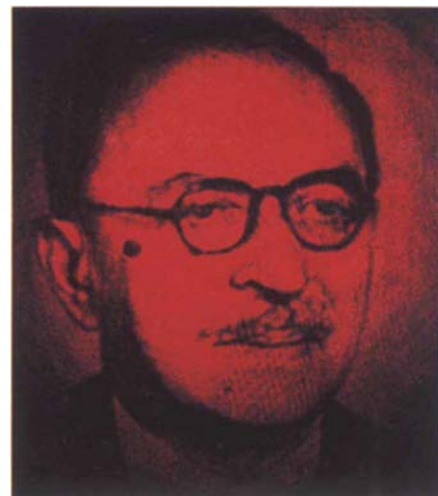


FIG. 2 Dennis Gabor, reconstructed from a 1-mm² peptide hologram. Gabor invented holography in 1948 (ref. 5).

around their long axis (see Fig. 2 on page 506). As a result, the asymmetry achieved in the film is higher than in traditional azo-dye doped polymers, resulting in higher refractive index changes, and consequently a higher storage capacity.

At this stage, the practical implications of these new peptides in holographic memories are difficult to predict, as the recording time is still long (several seconds) and we don't know whether they can record information in the thick films (of the order of 1 mm) needed in order to achieve high storage density. They do have the advantage that no electric field is required to write or read the information as in photorefractive polymers.

The high potential of organic molecular materials for optics has been confirmed. Such supramolecular structures will surely produce breakthroughs in the future. □

Nasser Peyghambarian and Bernard Kippelen are in the Optical Sciences Center and the Center for Advanced Multifunctional Polymers, University of Arizona, Tucson, Arizona 85721, USA.

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McGeorge Bundy (1919–96)

For years to come, historians will be writing, musing and speculating about the origins and originators of the US descent into the quagmire of Vietnam. Prominent among "the best and the brightest" being judged and second-guessed will be McGeorge Bundy, who died in Boston of a heart attack on 16 September, at the age of 77.

For years to come, historians will also be analysing and interpreting the critical events during mankind's perilous journey through the first 50 years of the nuclear age. And once again Mac Bundy will be found among those best and brightest who dealt with nuclear issues. He was a policy adviser at critical moments of decision such as the Cuban missile crisis of 1962. A brilliant scholar and prolific writer, he helped strengthen and shape public understanding, and influenced government policies on important nuclear arms control issues. I believe there will be a clear appreciation of the success of those best and brightest in creating what Tom Schelling has labelled "a tradition of non-use", lasting for 51 years.

Bundy graduated first in his class from Yale University as a mathematics major and subsequently switched to international relations as a junior fellow in the Harvard University Society of Fellows. After service in the Second World War, he became an assistant to Henry L. Stimson, who had served as secretary of war under Franklin D. Roosevelt, and collaborated in the preparation of Colonel Stimson's autobiography. This association involved close analysis of the US decision to drop the atom bomb on Hiroshima and Nagasaki — the beginning of Bundy's deep scholarly attention to matters of nuclear war and peace, danger and survival, that were an underlying theme throughout his life.

Returning to Harvard in 1949, he rose quickly to become dean of the faculty before moving to Washington in 1961 as national security adviser to John F. Kennedy. Following Kennedy's assassination in 1963, he remained in the same capacity under Lyndon Johnson until 1966.

During his years with Kennedy, two events occurred that had a lasting impact on future worldwide nuclear policy. The first was the Cuban missile crisis which brought the United States and the Soviet Union dangerously close to nuclear conflict. During the critical two-week period between discovery of the Soviet missiles in Cuba (by cameras

aboard a U2 aircraft) and their stand-down and removal by Khrushchev, Bundy was one of the small group in the Oval Office with Kennedy that stared the awesome possibility of a nuclear war straight in the eye.

As Bundy wrote 25 years later in the superb and authoritative *Danger and Survival: Choices About the Bomb in the First 50 Years*, "I have argued that the risk was small, given the prudence and the unchallenged final control of the two

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leaders, and I think that is the right retrospective judgment. But it is one thing to observe after a crisis is over that both sides were careful, and quite another to feel free to take such prudence for granted while the crisis lasts".

Mac Bundy's subsequent scholarship and writings on the dangers and risks to survival in a nuclear armed world bear the clear stamp of his harrowing experience of the Cuban missile crisis. It is the historian's dictum to learn from the lessons of the past in order to avoid repeating mistakes in the future. In the early dawn of the nuclear era, however, there was precious little relevant history to guide nations as to how to manage the risks, and avoid escalating local conflict into a worldwide nuclear holocaust. With his keen insight as an historian, Bundy plunged deeply into the analysis of the choices that governments made on critical nuclear issues. The understanding he gained formed the basis for *Danger and Survival*, a history to guide future generations.

It was also on Mac Bundy's watch as national security adviser, in 1961, that the Soviet Union broke the Eisenhower/Khrushchev moratorium on atmospheric nuclear testing. Negotiat-

ing the delicate path from there to the limited test ban treaty of 1963 was "a good first step that was achieved primarily by world opinion", as he wrote later. After that, his was one of the most eloquent voices in support of a comprehensive test ban treaty that would end nuclear testing once and for all.

Bundy participated in an appeal for such a treaty in one of his last public appearances, in Washington on 19 June of this year. A comment he made there reveals his pragmatic philosophy and his sustained commitment: "The step-by-step approach to the process of reducing nuclear danger has served us well. It has required attention to politics. It has required steadfastness in difficult seasons. It has required and it has had the strength that comes from an increasing community of those who pay steady and continuous attention to this issue". Sadly, Bundy died eight days short of witnessing the signing of that treaty by the five declared nuclear powers, now joined by most of the world's nations, at the United Nations on 24 September.

Following his years in Washington, Bundy returned to the private sector, as president of the Ford Foundation for 14 years, as chairman of the Population Council, and as professor of history at New York University for ten years. Subsequently, he assumed chairmanship of the Commission on Reducing Nuclear Danger at the Carnegie Corporation of New York.

During all that time, Bundy put the mark of his incisive thinking and eloquence on most of the critical nuclear issues of our time. His views on the challenges and opportunities that we face in the post-Cold-War world were summarized in *Reducing Nuclear Danger*, a treatise that he co-authored in 1993 with Admiral William Crowe, currently the US ambassador in London, and me.

It was my privilege to know Mac Bundy as a collaborator whose brilliance was matched by his mastery of the English word. He had the marvelous gift of distilling the essential ideas from lengthy, and at times arcane, discussions of nuclear policy, and expressing them in a lucid literary style. It was also my great privilege and pleasure to count Mac Bundy as a friend and to enjoy his humour, warmth and wisdom.

Sidney D. Drell

Sidney D. Drell is at the Stanford Linear Accelerator Center, Stanford, California 94309, USA.

Corbis-Bettmann/UPJ

Another green revolution

Steven G. Boxer

FOR most scientists, the first glimpse into the world of green-fluorescent protein (GFP) came early in 1994 with the publication of a striking image of a glowing, green nematode. The picture accompanied a paper by Chalfie and colleagues¹, which pointed to the stunning prospect of using fluorescence-imaging microscopy with GFP as a label for tracking the expression and location of other proteins within organisms as diverse as bacteria and nematodes. The three-dimensional structure of GFP just solved by Ormö *et al.*² and Yang *et al.*³, and reported in *Science* and *Nature Biotechnology* respectively, is equally stunning. The structure of GFP is so perfectly suited for its function it looks as if it were manufactured in a machine shop.

In nature, GFP is made by the jellyfish *Aequorea victoria*, which is widely found in the northwest Pacific Ocean. In the jellyfish, GFP functions in concert with the calcium-ion-activated protein aequorin. In response to shaking or attack, calcium-ion levels change and aequorin generates an excited state. The blue light from excited aequorin transfers to GFP, which then emits a bright green flash,

presumably blinding the attacker. The jellyfish aequorin-GFP system is also activated by ships moving through the water leaving a trail of light. This explains the long-standing interest of the US Office of Naval Research in GFP and bio-

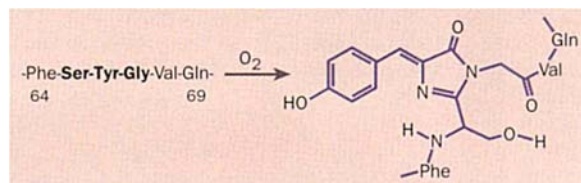


FIG. 1 Autocatalytic oxidation of GFP amino acids leads to the chromophore shown on the right. The green fluorescence requires further interactions of the chromophore with other parts of the protein.

luminescent organisms in general, a perfect example of science stimulated by military interest ultimately having a much wider impact.

Over the past 15 years, a group of scientists including William Ward at Rutgers University and Frank Prendergast at the Mayo Cancer Center has laboured in relative obscurity to characterize GFP

biochemically. Most coloured proteins contain a bound prosthetic group, such as retinal bound to opsin in the visual pigments, chlorophylls in photosynthetic systems, and haem which gives blood its red colour. These pigments can be extracted from their protein host; however, specific pigment-protein interactions alter the colour substantially, and this is often a key to their biological function. GFP is unusual as its pigment is derived from the post-translational oxidation of a bit of the polypeptide itself⁴ (Fig. 1).

The peptide sequence concerned (serine-tyrosine-glycine) is found in other proteins, but does not undergo the chemical transformation needed to form the pigment. This led some to suspect that the jellyfish possesses an elaborate enzymatic machinery to catalyse

the oxidation; but this is not the case, as became clear when Chalfie and co-workers¹ demonstrated that GFP could be expressed and the pigment developed in a wide variety of organisms. In fact, the chemical transformation that generates the pigment is an autocatalytic oxidation that can occur so long as most of the GFP polypeptide sequence is expressed and oxygen is present. No prosthetic group needs to be added.

Therein lies the power of the GFP system as a fluorescent label. Microscopists and pathologists routinely stain complex tissues to highlight particular features. During the past two decades, microscopy has been revolutionized by a remarkable collection of dyes used as *in vivo* indicators of physiologically important quantities such as pH, transmembrane potential or the concentration of calcium ions⁵. Proteins can be stained by binding to specific antibodies that can be imaged by a variety of techniques. All these methods, however, involve the addition of an exogenous agent. By contrast, the DNA sequence coding for the GFP polypeptide⁶ can be fused to that of any protein whose expression or location is of interest. Under favourable conditions, the expressed GFP domain folds independently of the protein to which it is fused

PALAEONTOLOGY

The 365-million-year-old grin

WHAT is the connection between a failed attempt to reach the North Pole by balloon, the political status of Greenland and the earliest amphibians? All is made clear in the long-awaited monograph on the Devonian tetrapod *Ichthyostega* by Erik Jarvik, published as an issue of *Fossils and Strata*

(40, 1-213; 1996). Although this animal has featured in textbooks for decades, this is, remarkably, the first detailed, complete description.

Jarvik's colleague, Gunnar Säve-Söderbergh, wrote a preliminary account of the fossil material in 1932, Jarvik undertaking the full description after Säve-Söderbergh's early death in 1948. After a long and distinguished career (he has been publishing since 1935), he has saved the best until last. Not everyone will agree with his interpretations, but the monograph will stand as a palaeontological resource for all time. *Ichthyostega's* welcoming



grin (above) appears on the front cover.

The fossils might have eluded discovery entirely were it not for the disappearance, in 1897, of Andrée Salomon's balloon-borne North Pole expedition. The search for survivors took geologists to East Greenland, where they found plenty of fossils, but no balloon. According to Jarvik, the discovery of *Ichthyostega* by a Danish-Swedish expedition may have been instrumental in the ruling in 1933, by the International Court of Justice in the Hague, to award Greenland to Denmark, rather than Norway.

Henry Gee

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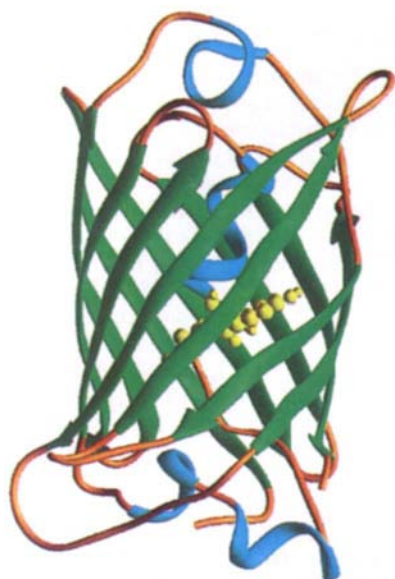


FIG. 2 The three-dimensional structure of wild-type GFP, adapted from Yang *et al.*³. The 11 β -strands comprising the barrel are shown in green; the caps at the top and bottom of the barrel are shown in tan; and the chromophore (yellow) is found in the middle of the barrel along the central helix shown in blue.

and accompanies it *in vivo* as a covalently attached fluorescent label.

The new three-dimensional structure of GFP is beautiful and unique (Fig. 2). Most of the polypeptide is folded into a tight, cylindrical, barrel structure consisting of a highly regular series of 11 β -strands. The outer diameter of the barrel is about 30 Å; the inner surface is quite polar and can accommodate the single α -helix found neatly packed inside. The sequence from which the chromophore is derived is contained right in the middle of this α -helix. The barrel is capped on both top and bottom, so the chromophore is packaged in an environment entirely comprised of the protein and some structured interior water molecules, completely protected from the surroundings.

Despite the nearly perfect engineering achieved by the jellyfish, several investigators have sought to improve upon the properties of GFP for applications in biotechnology. Native GFP has two absorption maxima, a strong peak at 395 nm and a weaker peak around 477 nm. These peaks appear to be associated with two conformational isomers of the chromophore that can interconvert both in the ground and excited state. On exposure to light, the form corresponding to the peak at 395 nm photoconverts into the form absorbing around 477 nm. The excited-state process appears to involve ultrafast proton transfer followed by much slower solvation⁷.

The two forms and the interconversion process can now be explained by specific interactions between the chromophore and amino acids on the inner surface of the β -barrel. The two published structures

are actually of slightly different proteins — a mutant, in which the serine at residue 65 is converted to threonine, from Ormö *et al.*²; and the wild type from Yang *et al.*³. Remarkably, the replacement of serine 65 with threonine greatly simplifies the spectrum to a single peak absorbing at 488 nm (ref. 8). Further engineering of the surrounding interior surface of the barrel, remote in primary sequence from the chromophore, leads to optimization of the chromophore properties for use in fluorescence microscopy. Until now, these changes were largely based on inspired guesses or screening large libraries of mutants, but the palette of colours can now be rationally designed based on the structure.

This last feature and the observation of ultrafast excited-state dynamics⁷ suggest that GFP is an ideal object to test and refine computational methods widely used to analyse and re-engineer proteins and to characterize binding sites. The GFP chromophore by itself does not exhibit

green fluorescence; rather, a specific set of interactions with the protein 'solvent' leads to the unique spectral and dynamic properties. In fact, GFP can be reversibly denatured with a loss of the green fluorescence⁹. This dependence of the colour on tertiary structure might be turned to advantage for studying protein folding in real time.

Finally, a key problem in biology is discovering which proteins interact at a molecular level during development and self-organization. GFP tags may be useful for identifying which proteins are in close proximity by energy transfer using differently coloured GFPs or fluorescence depolarization. Thousands of laboratories are now reputed to be using GFP for a wide range of clever purposes, so the future looks very green. □

Steven G. Boxer is in the Department of Chemistry, Stanford University, Stanford, California 94305-5080, USA (e-mail: SBoxer@Leland.Stanford.edu).

REPRODUCTIVE BIOLOGY

The vocabulary of the egg

Roger Gosden

At one time, eggs were regarded like the cargo in the hold of a vessel — finally discharged after a voyage by ovulation or, more frequently, shipwrecked in atresia. We now recognize that each egg actively influences the development of its own follicle — it despatches commands affecting the growth and differentiation of the granulosa cells around it, while receiving information and nutrition from them¹. The extent to which the character or quality of an egg shapes the destiny of its follicle is still open to question. But there is no longer any doubt that the phenotype of the follicle is affected by signals from the oocyte inside, and on page 531 Dong *et al.*² report the first identification of such an oocyte factor.

The earliest experimental indication that oocytes influence follicle development was published more than 25 years ago by Andy Nalbandov and his colleagues at the University of Illinois³. They showed that when oocytes were removed from Graafian follicles in rabbit ovaries, the granulosa cells became prematurely luteinized — that is, they behaved as though they were post-ovulatory, and secreted progesterone. Any questions about the artefactual nature of these results were quashed by studies showing that oocytes secrete a factor (or factors) that increases the production of oestro-

gen and decreases that of progesterone by granulosa cells that have been stimulated by follicle-stimulating hormone (FSH) and testosterone *in vitro*⁴.

So the idea that oocytes communicate with neighbouring cells is not new, nor should it be particularly surprising in an integrated developmental unit (Fig. 1). But the range of their vocabulary is greater than had been suspected. After removal of the oocyte, the surrounding cumulus granulosa cells within a large follicle can no

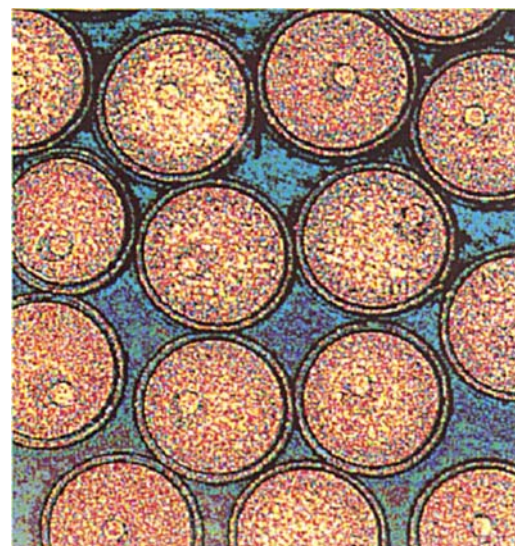


FIG. 1 A cluster of fully grown mouse oocytes: each cell is enclosed by a zona pellucida and contains a prominent nucleus and nucleolus. (Photo: J. J. Eppig, Jackson Laboratory, Bar Harbor, Maine.)

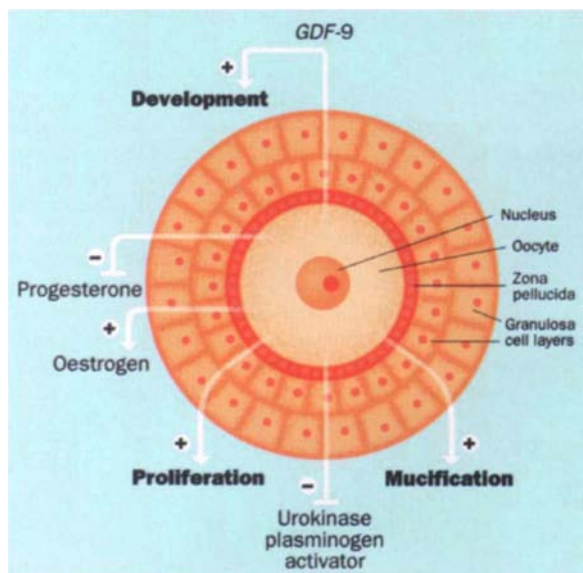


FIG. 2 The oocyte secretes a number of factors (not necessarily simultaneously) that influence the surrounding granulosa cells and their ability to respond to follicle-stimulating hormone. Dong *et al.*² have now identified GDF-9, an oocyte-derived factor that is required for somatic cell development *in vivo*.

longer secrete hyaluronic acid in response to FSH or cyclic AMP⁵, at least in mouse ovaries. So they fail to produce a sticky mucinous mass that can be picked up after ovulation by the ciliated entrance to the fallopian tube. What is more, as well as the so-called 'cumulus-expansion-enabling factor', oocytes secrete something that helps to prevent the breakdown of the extracellular matrix by inhibiting the production of urokinase plasminogen activator⁶. Oocytes also stimulate the proliferation of granulosa cells throughout the course of follicle growth. After oocyte removal, cultured granulosa cells from small follicles and cumulus masses from large ones cannot incorporate as much tritiated thymidine as can intact controls⁷, and are growth-retarded as a consequence. Such findings are reminiscent of the morphology of polyovular follicles in which each oocyte can induce the formation of its own mound of cumulus cells.

But the quest to isolate and identify the factors responsible for these phenomena has been frustrating, perhaps because they are rapidly degraded under culture conditions. Nevertheless, the experimental evidence leaves little doubt that several factors are involved. And because granulosa cells respond to an oocyte-conditioned medium in a concentration-dependent manner, and they do not require physical contact with the oocytes, the active agents are evidently secreted, rather than simply being passed from cell to cell through gap junctions.

Dong *et al.* have now shown² that a specific oocyte product, growth differentiation factor-9 (GDF-9), is required for the development of granulosa cells (Fig. 2). Targeted deletion of this member of the transforming growth factor- β superfamily

using embryonic stem-cell technology, leads to sterility in homozygous female mice ($gdf9^{m1}/gdf9^{m1}$) — in contrast, homozygous males are normally fertile. The defect is independent of the expression of FSH, luteinizing hormone or activin type-II receptors, and reveals that the primary-follicle stage is a critical transition in development. Oocytes in mutant mice grow and secrete a zona pellucida, but follicles form only one layer of granulosa cells and the theca fails to differentiate. The gonads remain diminutive and, as a result of weak secretory activity, serum gonadotrophin levels rise and encourage the growth of ovarian cysts.

What, if anything, is the practical significance of these findings? For one thing, GDF-9 may help to overcome deficiencies in follicle-culture technology. There is even a slim chance that it will turn out to be the aforementioned mucification factor, although there is evidence against this view⁸. The GDF-9 knockout mouse could also become a useful model for clinical pathology. Resistant-ovary syndrome is a heterogeneous disorder of early follicle development in women, and in some cases it could be due to a deficiency in GDF-9. One thing is clear — GDF-9 is unlikely to have much impact on the rate of follicle attrition during ageing, because it acts downstream of the primordial stage in which follicles are stored pending recruitment for growth. But the discovery of the dramatic effects of this gene will encourage more efforts to identify other oocyte-derived factors affecting follicle development. This could throw fresh light on unexplained forms of infertility and indicate new strategies for contraception. □

Roger Gosden is at the Centre for Reproduction, Growth and Development, University of Leeds, Division of Obstetrics and Gynaecology, Clarendon Wing, Leeds General Infirmary, Leeds LS2 9NS, UK.

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Eschewing the fat

THE British government has set a target for reducing obesity. In effect, the nation is being collectively asked to shed about 20,000 tons of fat by 2005. Progress so far has been negative.

Obesity seems to start in childhood, when our fat cells readily multiply. Thereafter they are always ready to be filled, and rapidly undo the most dedicated slimming efforts. Liposuction can remove them permanently, of course; but a government liposuction initiative would not be popular. Musing on the problem, Daedalus recalled that the anaesthetic gas nitrous oxide is over five times more soluble in fat than it is in water. Hence its use as a blowing agent in aerosol cans of whipped cream. So if you pressurized an obese patient in nitrous oxide, the gas would dissolve preferentially in his fatty tissue. If the pressure were suddenly released, it would bubble out of solution. His fat cells, loaded with dissolved gas, would swell up and burst. His other cells, containing very little fat, would be unaffected.

This, of course, is a form of 'the bends', that painful syndrome felt by a diver who comes up from deep water too quickly. DREADCO's 'fat-bending chamber' will minimize this discomfort. Its content of oxygen and nitrous oxide will anaesthetize the subject during decompression. The smallest feasible pressure-drop will be used, probably from somewhat above normal pressure to somewhat below it, so as to destroy only a small fraction of the subject's fat cells. Their broken fragments and freed fat will be released into the body; it may take a few days for the liver and the body's scavenging systems to dispose of this detritus. The subject can then have a further session. For the government mass health programme, Daedalus is designing a mobile collective fat-bending chamber, a sort of pressurized bus to tour obesity trouble-spots, and in which dozens of people can have their fat bent away simultaneously.

Cosmetic fat-bending poses trickier problems. How to remove unwanted fat, while leaving more desirable deposits untouched? Daedalus recalls the old technique of 'cupping' — reducing the pressure over a small area of the skin by a sort of suction bell-jar. He plans to develop it into a controllable, local method of fat-bending. The patient would be anaesthetized with nitrous oxide; then in slow, carefully judged stages, fat would be cupped away from the regions of excess. The client would slowly acquire the figure of his or her dreams. It would be permanent: stable against the most wanton bingeing. The fat cells which might store the deadly calories would simply no longer be there. David Jones

Caribbean sea-fan mortalities

SIR — Epizootics resulting in mass mortalities of Caribbean sea fans have been observed for more than 15 years, but the cause has remained unresolved^{1–3}. Here we report for the first time the presence of a putative pathogen associated with diseased sea-fan tissue throughout the Caribbean.

We obtained samples of healthy and diseased *Gorgonia ventalina* and *G. flabellum* (Cnidaria: Gorgoniidae) from the Bahamas, the British Virgin Islands, Curaçao, Saba and Trinidad⁴. We examined

only one type of fungus was common to all diseased tissue. Microscopic observations of pure cultures grown on Hektoen Enteric agar (HE, Difco), supplemented with artificial sea water, showed the presence of conidiophores similar to those produced by the genus *Aspergillus*⁶.

We used one of these isolates, SA-25 (from Saba), as an inoculum onto healthy *G. ventalina* (from San Salvador, Bahamas) in aquaria. Within 3 days, the area inoculated with SA-25 showed symptoms of the disease (recession of the rind, see figure).

Microscopic observation of affected tissue showed abundant hyphal growth at the edge of the receding rind. We inoculated affected tissue onto HE medium, and found that fungal isolates arising from this inoculum are identical to SA-25. We did not find this isolate in 'control' areas, which included inoculated areas with the HE medium on which the isolate was grown, a different fungal isolate (from a diseased Trinidad sample), and a mock inoculation with a sterile loop (to check for injury).

We demonstrated that the disease is transmissible in San Salvador, Bahamas by removing diseased tissue from infected sea fans and inserting it into cuts made on healthy stands. We compared carbon source utilization patterns^{7, 8} among the disease isolates SA-25,

SS-7, seven reference strains (each a different species of *Aspergillus*) and two reference species of *Penicillium*. Based on these patterns, we found that the sea-fan isolates are more similar to each other than to the *Aspergillus* species. Nucleotide sequence analysis of the 18S ribosomal RNA gene confirms that the isolate, SA-25, is more similar to *Aspergillus fumigatus* than to any other species available in the GenBank database. Sequences showed, however, only 96% identity.

Thus, morphological, physiological and nucleotide-sequence data all indicate that the putative pathogen falls within the genus *Aspergillus* and is likely to represent a new species. *Aspergillus* is not a common marine fungus, but it is a typical soil inhabitant. It generally does not sporulate in aqueous environments. Similarly, our isolates did not sporulate when covered with sterile artificial sea water and we saw

only hyphae (no spores) in diseased rind tissue. This indicates that the primary infection occurs by hyphae, perhaps associated with sediment particles. Persistent mortality, then, would require a continuous source of infection. This situation would exist in areas receiving increased sedimentation loads due to the loss of forested lands².

Garriet W. Smith

Lisa D. Ives

Biology Department,
University of South Carolina at Aiken,
South Carolina 29801, USA

Ivan A. Nagelkerken

Carmabi Foundation/Curaçao

Underwater Park,
PO Box 2090, Piscaderabaai z/n,
Curaçao, Netherlands Antilles

Kim B. Ritchie

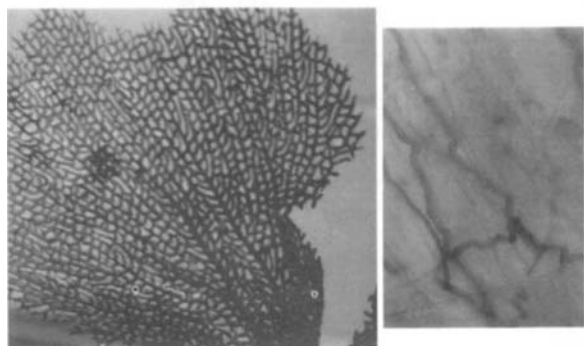
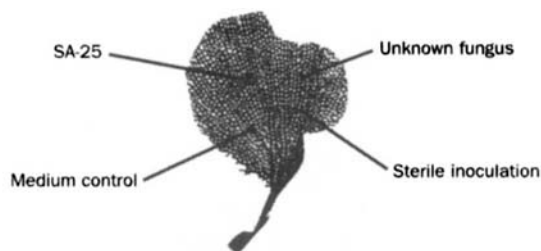
Biology Department,
University of North Carolina,
Chapel Hill,
North Carolina 29599-3280, USA

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Vesicle assembly in microtubes

SIR — Cytomimetic microstructures are attracting much attention¹, with most studies being focused on spherical structures such as vesicles and liposomes². Here we report the non-covalent self-assembly of vesicles encapsulated by microtubular structures made up of synthetic oligoglycine-based bola-amphiphiles.

The bola-amphiphiles $C(n)[G_m]_2$ ($n = 6$ and 10, $m = 2$ and 3; Fig. 1a) are dicarboxamides with two oligoglycine head groups at each end. They form white solids (melting points $> 210^\circ\text{C}$) with very low solubility in water, whereas the sodium salts are very soluble ($\leq 15\text{ mM}$). If an aqueous solution (concentration 10 mM, pH 7–8) is kept continuously at room temperature for 2–3 weeks, bola-amphiphiles form very thin fibrous assemblies. Phase-contrast microscopy of $C(10)[G_3]_2$ reveals well-defined tube-like fibre structures with a uniform diameter (about 1–2 μm), containing many vesicular assemblies (Fig. 1a). The spheres we observe strongly suggest the existence of multilamellar vesicles³. The fibres are not worm-like tubules



Sea-fan inoculation experiment. Top, sea fan inoculated with the putative pathogen SA-25, an unknown sea fan fungus, HE medium (on which SA-25 was grown) and a sterile inoculation. Bottom left, lesion produced by SA-25. Bottom right, micrograph of receding rind from the lesion showing fungal hyphae.

samples preserved in ethanol (80–100%) under the microscope. Diseased samples showed distinct recession of rind tissue (coenenchyme, the outer organic rich matrix containing the living polyps), exposing the axial skeleton (dead central core). The exposed core was often colonized with a cyanobacterium similar to *Phormidium corallyticum*, the organism responsible for black-band disease in hard corals⁵. Although this colonization of the core on some samples was extensive, it appeared secondary to the recession caused by the fungus. In all diseased samples, we observed hyphae embedded in the receding edge of the rind. Hyphae were not seen in healthy rind tissue.

We compared bacterial and fungal isolates obtained from healthy and diseased samples from each site after subculturing on similar media (reciprocal plating). Based on colony and cellular morphology,

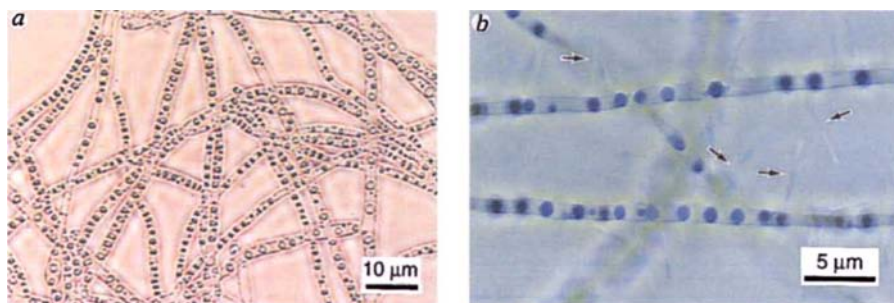


FIG. 1 Molecular structure of oligoglycine-based bola-amphiphiles $C(n)[G_m]_2$. Vesicle-enclosing tubular organic fibres based on a, $C(10)[G_3]_2$ by phase-contrast light microscopy; and b, $C(10)[G_2]_2$ by video-enhanced high-powered dark-field light microscopy. Arrows denote thin needle-like crystals projecting from the periphery of the tubes.

(so-called 'myelin figures'⁴) but are apparently stiff tubes: a few minutes of bath-type sonication has no observable effect on their morphology. Video-enhanced dark-field microscopy clearly shows that the tube structures coexist with spherical and ellipsoidal vesicles inside them (Fig. 1b). There are smaller spherical vesicles around the ends of the fibres, which undergo brownian motion inside the tubes. We saw no vesicles in the external surrounding aqueous medium.

We found no observable change of morphology after isolation of these structures, dehydrating them and completely drying them in a high vacuum. The structures can be reconstructed by rehydration of the dried fibres by addition of excess water and sonication. Most of the ends of the tubes are open to the aqueous medium after rehydration — the formerly closed ends are apparently destroyed. We

saw several floating vesicles outside the tubes, having apparently leaked out from the open ends.

The reduction of one glycine residue at both ends in the bola-amphiphile $C(10)[G_2]_2$ produces apparently thinner fibres from which very thin needle-like crystals project at the periphery (arrow, Fig. 1b). In contrast, reduction of the hydrocarbon bridge from C-10 to C-6 in $C(6)[G_2]_2$, or replacement of the glycine residues with other *N*-substituted amino acids such as proline or *N*-methylglycine, does not produce the tube structures.

Both the fibrous microtubes and the enclosed vesicles are probably composed of monolayered unilamellar or multilamellar membranes⁵⁻⁷. The Fourier transform infrared (FTIR) spectrum for the dehydrated tubes of $C(10)[G_3]_2$ has a C=O stretching band at $1,743\text{ cm}^{-1}$, suggesting the existence of a $-\text{COOH}\cdots\text{OOC}-$ acid-anion dimer (Fig. 2a, b). This means that the terminal carboxylate anion is gradually protonated during ageing, perhaps by the dissolution of carbon dioxide in air. FTIR spectroscopy also demonstrates the existence of bifurcated hydrogen bonds between the two carboxylic acids in the outer needle-like crystals (Fig. 2c). Thus, the delicate balance and the networks of the hydrogen bonds contribute to the unique formation of the vesicle-enclosing microtube structures as well as to the growth of needle-like crystals on their outer walls.

Toshimi Shimizu*, Masaki Kogiso Mitsutoshi Masuda

Department of Organic Materials,
National Institute of Materials and
Chemical Research,
1-1 Higashi, Tsukuba, Ibaraki 305, Japan
e-mail: tshmz@ccmail.nimc.go.jp

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*To whom correspondence should be addressed.

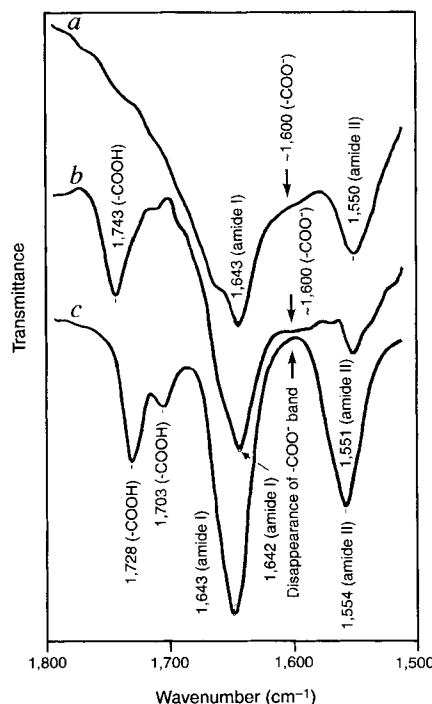


FIG. 2 FTIR spectra of the bola-amphiphiles in the carbonyl stretching regions. a, Sodium salt of $C(10)[G_3]_2$; b, dehydrated dry microtubes of $C(10)[G_3]_2$; c, single crystals of $C(10)[G_2]_2$ generated from the outer wall of the tubes.

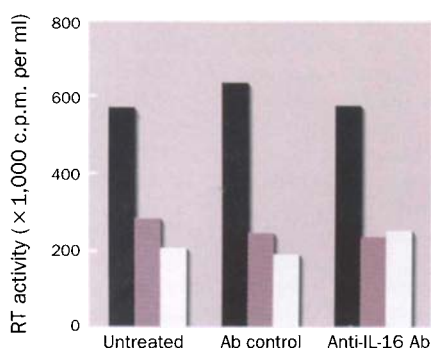
Role of IL-16 in HIV replication

SIR — Baler *et al.* have reported that the previously identified cytokine, lymphocyte chemotactic factor¹ (now designated interleukin-16, or IL-16), has inhibitory activity against HIV replication in peripheral blood mononuclear cells (PBMCs)². They isolated IL-16 from concanavalin A-stimulated PBMCs from African green monkeys (IL-16_{AGM}) and demonstrated that a recombinant form of the primate IL-16 is highly homologous to human IL-16, differing in only seven amino acids. Recombinant forms of these molecules were shown to inhibit HIV replication in PBMCs depleted of CD8⁺ cells in a dose-dependent manner. IL-16_{AGM} could suppress HIV production at 10 ng per ml or greater, whereas 1,000 ng per ml or more of human IL-16 was required for any appreciable antiviral effect to be detectable. The authors suggested that IL-16 may be related to the previously described immunodeficiency virus suppressing lymphokine³ produced by primate CD8⁺ cells and may contribute to the low viral load and healthy clinical state of naturally infected African green monkeys and asymptomatic HIV-infected individuals.

We undertook a series of experiments to determine if the anti-HIV activity of CD8⁺ cells from HIV-infected individuals mediated by a CD8⁺ cell antiviral factor (CAF)^{4,6} is due to IL-16. First, we measured the level of IL-16 in 14 different CD8⁺ T-cell supernatants, seven of which contained CAF. IL-16 was quantitated by a sandwich ELISA⁷. In addition, we measured IL-16-mediated chemotaxis using a modification of a Boyden chamber method with purified nylon wool non-adherent T cells as target cells placed in the upper chamber of a 48-well microchemotaxis chamber^{7,8}.

The CD8⁺ cell culture fluids containing CAF could suppress HIV-1_{SF2} replication in activated CD4⁺ cells by $\geq 50\%$ when tested at a 50% dilution⁵. Two of seven CAF-positive fluids had detectable levels of IL-16 (335 and 1,115 pg per ml); these fluids also showed IL-16-mediated chemotactic activity. Four of seven CAF-negative fluids showed the presence of IL-16 (13–469 pg per ml) with associated chemotactic activity. The amount of IL-16 protein found in these CD8⁺ cell supernatants did not correlate with the presence of CAF-mediated antiviral activity.

Next, we assessed the anti-HIV activity of recombinant human IL-16 in the assay routinely used to measure CAF activity⁵. We cultured purified CD4⁺ cells, acutely infected with HIV-1_{SF2} (the same HIV isolate used in ref. 1), in the continual presence of IL-16 at different concentra-



Effect of anti-IL-16 neutralizing antibodies on CAF activity. CAF-positive culture fluids (grey and white bars) and control medium (black bars) were left untreated or treated for 30 min with 5 μ g per ml of control antibody or neutralizing antibody specific for IL-16, before their addition to HIV-1_{SF2}-infected CD4⁺ cell cultures. The extent of HIV replication, as indicated by the amount of particle-associated reverse transcriptase (RT) activity in the culture fluids, was monitored every 2 days⁵. The anti-recombinant human IL-16 neutralizing monoclonal antibody recognizes the C-terminus of the secreted 130 amino acid biologically active IL-16 molecule. This antibody (5 μ l) neutralizes 90% of the biological activity of 50 ng per ml recombinant human IL-16. As described previously, this antibody also neutralizes natural IL-16 chemotactic activity derived from T-cell supernatants⁸ or biological fluids⁷ (for example, bronchoalveolar lavage fluids).

tions, and monitored virus production. We saw only a limited suppressive effect of IL-16, with a 45% reduction of particle-associated reverse transcriptase activity in the culture fluid at only the highest concentration tested, 5 μ g per ml, similar to the earlier results². Moreover, a neutralizing antibody specific for human IL-16 (at concentrations that neutralized all the chemotactic activity of 50 ng per ml recombinant human IL-16) did not block the antiviral activity of CD8⁺ T-cell supernatants against CD4⁺ cells acutely infected by HIV-1 (see figure).

Some concern has been raised in Scientific Correspondence^{9,10} that the 130-amino-acid recombinant IL-16 molecule¹ represents only a portion of a much larger, naturally produced precursor protein. This possibility questions the true nature and functional role of IL-16. However,

purified natural IL-16 (relative molecular mass 17,000 (17K) as detected by SDS gels) and recombinant IL-16 (17K) exhibit all the functional properties attributed to IL-16/LCF (refs 1, 2, 11). These include chemotactic activity, induction of IL-2 receptor expression and inhibition of HIV replication (at high concentrations). Regardless of the true nature of IL-16, the antibody used in the ELISA and neutralization assays reacts with both the naturally produced IL-16 and the recombinant protein¹. Thus, we conclude that the factor-mediated anti-HIV activity

of CD8⁺ T-cells which has been described in HIV-infected individuals⁴⁻⁶ is due to a factor other than IL-16.

Carl E. Mackewicz

Jay A. Levy

Department of Medicine,
University of California School of Medicine,
San Francisco, California 94143-1270, USA

William W. Cruikshank,

Hardy Kornfeld

David M. Center

Department of Medicine,
Boston University School of Medicine,
Boston, Massachusetts 02118, USA

Mechanosensors in plants

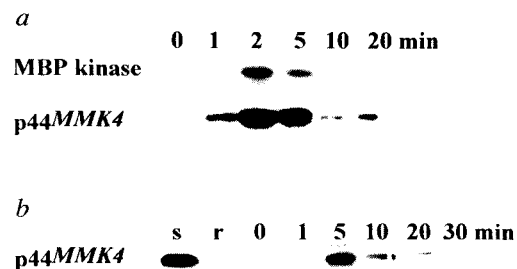
SIR — Mechanical stresses in the form of touch, rain and wind are important environmental signals in plants, inducing various physiological responses¹. Although it has recently been shown that calcium fluxes occur in response to touch and wind², and several genes encoding calmodulin-like, calcium-binding proteins and protein kinases become transcriptionally induced^{3,4}, the molecular mechanism of mechanosensing in plants is not well understood. To investigate a potential role of protein kinases in the signal transduction of mechanical stimuli in plants, we have used in-gel kinase assays of mechanically stimulated alfalfa leaves (a in the figure).

As shown by the ability to phosphorylate myelin basic protein (MBP), a protein kinase of relative molecular mass 44,000 (44K) becomes activated within 1 minute after a mechanical stimulation of 2 seconds. The activation of the MBP kinase is transient and disappears after 10 minutes. The molecular mass of the MBP kinase and its ability to phosphorylate MBP suggested to us that the kinase might belong to the class of mitogen-activated protein (MAP) kinases.

To test this hypothesis, we prepared protein extracts from mechanically stimulated leaves and immunoprecipitated them with M7, M14 and M17 antibodies raised against the carboxy-terminal 10 amino acids of the alfalfa MAP kinases MMK4, MMK2 and MMK3⁵. We found that active kinase was immunoprecipitated only with M7 antibody, which 'decorates' a single 44K protein in immunoblots of alfalfa cells⁵. This evidence, together with the observation that the activity of immunoprecipitated p44^{MMK4} kinase (see figure) changes with similar kinetics to the kinase detected in the in-gel assay, suggests that

mechanosensing in plants involves a MAP kinase pathway.

In contrast to leaves, where p44^{MMK4} kinase is only transiently activated on mechanical stimulation, we observed constitutive activation of the kinase in cells cultured in suspension (b in the figure, s). We suspected that this might be due to the constant agitation of the cells producing shearing forces between the cells and between the cells and the wall of the container. We therefore removed constantly shaken cells that had been cultured in a thin liquid layer in Petri dishes from the shaker and allowed them to rest for 1 hour (b in the figure, r). We then agitated



a, p44^{MMK4} kinase is activated by mechanical stimulation of alfalfa leaves. Leaves on intact plants were mechanically stimulated for 2 s. At 0, 1, 2, 5, 10 and 20 min after treatment, leaves were collected and immediately frozen in liquid nitrogen. After protein extraction, protein kinase reactions were performed either in the gel (upper panel) or as immune kinase reactions (lower panel)⁵. For in-gel assays, each lane contained 20 μ g of total protein from leaf extracts, which was separated by SDS-PAGE containing MBP as a substrate. After renaturation, the kinase reactions were carried out in the gel. The p44^{MMK4} immune kinase assays were performed after immunoprecipitation of leaf extracts containing 100 μ g of total protein with protein A-purified M7 antibody, produced against a synthetic peptide encoding the carboxy-terminal 10 amino acids of the alfalfa MMK4 kinase⁵. p44^{MMK4} kinase activity was determined by protein kinase reactions with myelin basic protein and [γ -³²P]ATP. Phosphorylation of MBP was analysed by autoradiography after SDS-PAGE. b, Mechanical stimulation of suspension cultured cells activates the MMK4 kinase. Protein extracts from constantly shaken cells (s), cells allowed to rest for an hour (r), and from cells that were agitated for two seconds after rest (0–30 min) were used for p44^{MMK4} immune kinase assays as described above.

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the cells for 2 seconds and allowed them to rest again. At different times after these treatments, we prepared cell extracts from aliquots of these cells and analysed them by immune kinase assays for the determination of activity levels of p44^{MMK4} kinase.

In contrast to constantly shaken cells, which contain active p44^{MMK4} kinase (s), we detected no active p44^{MMK4} kinase in the 'rested' cells (r). After agitation, however, p44^{MMK4} kinase activation became apparent within 5 minutes (see figure). The activation is transient, because kinase activity decreases to nondetectable levels at 30 minutes after the mechanical agitation (see figure), indicating that short-term mechanical stimulation produces only a transient activation of the MAP kinase pathway. The constitutive activation of p44^{MMK4} kinase probably results from the continuous restimulation of the pathway.

The very fast activation of p44^{MMK4} kinase after mechanical stimulation suggests that the activation of the MMK4

pathway must be one of the cell's immediate responses to this stimulus. Although it is unclear what factors might be responsible for activation of the MAP kinase pathway, mechanosensitive ion channels have been described in several organisms⁶⁻⁸ and could be involved in mediating the primary signals. Moreover, mechanical stimulation of plant cells induces rapid fluxes of calcium ions², suggesting that this ion may be important in mechanosignalling.

L. Bögre, W. Ligterink

E. Heberle-Bors, H. Hirt

Institute of Microbiology and Genetics, Vienna Biocenter,

Dr. Bohrgasse 9, 1030 Vienna, Austria

e-mail: hehi@gem.univie.ac.at

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Old head on young shoulders

SIR — A complex head is a fundamental defining feature of the vertebrates, but the origins and antiquity of many characters within the head are obscure. These can be investigated by comparison with cephalochordates (amphioxus), which retain characteristics of vertebrate ancestors^{1,2}. Here we report the developmental expression of *AmphiOtx*, the amphioxus member of the *Otx* homeobox gene class. *Otx* genes are essential for the formation of anterior head regions in the mouse^{3,4},

with conserved embryonic expression patterns across higher vertebrates^{5,6}.

Whole-mount *in situ* hybridization to amphioxus (*Branchiostoma floridae*) embryos reveals that the temporal and spatial expression pattern of *AmphiOtx* has intriguing similarities with that described for the vertebrate *Otx* gene family. Widespread expression in anterior tissues of vertebrates at early developmental stages is mirrored in amphioxus by strong early expression in anterior neur ectoderm and mesendoderm (a in the figure). In both taxa there is a sharp posterior expression boundary in the neur ectoderm, which stays in register with the underlying mesendoderm, suggesting a common neural induction/maintenance mechanism. At later developmental stages, *Otx* gene expression becomes restricted to specific cell groups in the anterior neural tube of both vertebrates and amphioxus (b–d in the figure): comparison of these cell types is particularly informative.

AmphiOtx expression is detected in the anterior tip of the cerebral vesicle, in the frontal eye region (containing putative receptor cells and future pigment spot), ventral to the neuro-pore. This is comparable to the conserved expression of vertebrate *Otx* genes in the

eyes, including the retina. An ancient role for this gene family in vision-associated cells is also supported by the requirement of the *Otx* homologue, *orthodenticle*, for the specification of the *Drosophila* eye⁷.

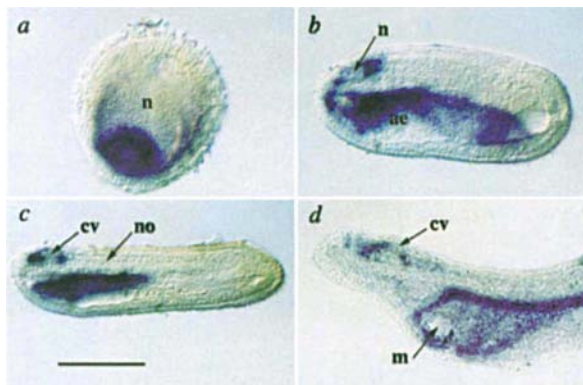
AmphiOtx is also expressed in the cerebral vesicle at the anterior margin of the floor plate, a region known as the infundibular organ (IO). This expression site is intriguing, as this organ is the source of Reissner's fibre; an acellular fibre in the chordate neural canal implicated in neuronal survival⁸. Although the source of this fibre is dorsally located in adult vertebrates, in the subcommissural organ (SCO) in the diencephalic roof, it is initially produced in the ventral flexural organ at the anterior margin of the floor-plate in at least some embryonic vertebrates⁹. Both regions express *Otx* during embryogenesis; thus, regulatory gene expression and function concur in suggesting the IO and the diencephalic SCO have a common evolutionary origin.

Gene expression is a useful clue to evolutionary homology, but care must be taken to distinguish different hierarchical levels of homology (for example, positional versus structural homology)¹⁰. In the *Otx* case reported here, we argue for homology of region and differentiated characters. We suggest that the conserved early deployment of *Otx* regulatory genes in the anterior mesendoderm and overlying neur ectoderm of vertebrates and cephalochordates indicates a common gene regulatory network between these two taxa. The later conserved neural expression of *Otx* genes suggests to us that the cephalochordate frontal eye is an unpaired homologue of the vertebrate paired eyes and that the ventral IO is homologous to the dorsal SCO. These assignments are consistent with a hypothesis proposed on the basis of cellular architecture¹¹. Our results imply that a distinct differentiated forebrain evolved before the separation of cephalochordates and vertebrates, predating skeletal tissues, migratory neural crest and elaboration of the vertebrate genome.

Nic A. Williams, Peter W. H. Holland

School of Animal and Microbial Sciences, The University of Reading, Whiteknights, Reading RG6 6AJ, UK

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Whole-mount *in situ* hybridization using a digoxigenin-labelled *AmphiOtx* riboprobe. a, Dorsal view of an early neurula stage (7 hour), anterior at bottom left, showing strong expression in the anterior neural plate and underlying mesendoderm. A lower level of expression is detected in the lateral endoderm. b, c, Mid and late neurula stage (14 hour and 18 hour) with expression localized in the cerebral vesicle and anterior endoderm. Anterior to left, dorsal at top. d, Rostral end of early larval stage (36 hour) with neural expression confined to two clusters of cells in the anterior cerebral vesicle. A few ectodermal cells just anterior to the cerebral vesicle also stain; endodermal expression persists in the anterior pharynx. cv, anterior cerebral vesicle; ae, anterior endoderm; m, forming mouth; no, notochord; n, neural plate or tube. Scale bar: a, 75 µm; b, 100 µm; c, 150 µm; d, 50 µm.

Walks on the wild side

David E. Allen

Bright Paradise: Victorian Scientific Travellers. By Peter Raby. Chatto & Windus: 1996. Pp. 276. £20.

SOMEONE really ought to make a special study of the debt owed by science to the alluring diversity of the order Coleoptera. It is well known that Charles Darwin had a beetle-collecting craze before the *Beagle* voyages, but less so that it was the same shared passion that first brought together Alfred Russel Wallace and Henry Walter Bates, the two great explorers of the Amazonian faunas. This is but one of numerous *trouvailles* to be gleaned from Peter Raby's extremely readable book.

Just as Lytton Strachey discovered a rich literary seam by quarrying the piously massive 'life and letters' tomes of which the Victorians were so fond, so Raby has boiled down into a more digestible form the multivolume adventures of the travellers and explorers of that period, rescuing these narratives from the near oblivion into which they have fallen.

On the argument that "the investigation of nature was the great nineteenth-century work", he has chosen scientific travel as his ostensible binding theme, although he has pardonably strayed from that in one or two cases where the motive seems rather to have been exploration pure and simple. Foremost among these is that of the remarkable Richard Lander, a Cornish innkeeper's son, who penetrated parts of the West African interior, where many grandly sponsored and well-provisioned expeditions had earlier come to grief.

It did not necessarily follow that scientific minds functioned scientifically when they arrived in untrodden parts, however. Francis Galton, of all people, showed next to no interest in the fauna and flora on his pioneering trip to southwest Africa, returning (in Raby's words) "as narrowly English and imperial in his attitudes as he was when he set out".

Fortunately, there were others with rather more curiosity and very much more persistence, some of whom spent years on end in the remotest of regions with little or no contact with civilization, let alone with like-minded travellers. The botanist Richard Spruce, for example, one of a trio whose Amazonian wanderings in search of specimens are grippingly recalled afresh, was away from home for as long as fourteen years without a break. Bates, his zoologist counterpart, was absent for a scarcely less heroic eleven. They had to cope with fever, endless torment by insects, hostile natives, dangerous animals, and at times a breakdown in communications with the

outside world so extreme that their clothes were reduced to rags and their money ran so short that they were lucky not to starve.

Wallace, Bates and Spruce were comparatively unusual in subsisting as freelances, gambling on finding and sending safely back enough that was rare or novel to earn them sufficient from the natural-history dealers in London to enable them at least to survive and cover their expenses. It was a slow way of making a living, as Raby drily remarks: at one point one of them had less than £27 to show for almost as many months of extremely assiduous collecting.

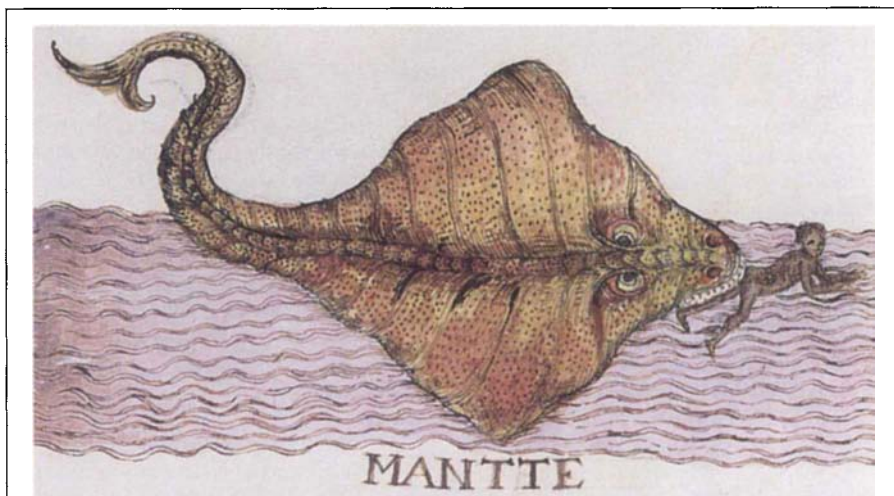
More typically, work of this kind took place under some form of official sponsorship, either directly, like many expeditions sent out after 1735, under the auspices of one government or another, or indirectly, by attachment as an individual to the crew of a naval vessel. Darwin was lucky enough to be engaged as one of those gentleman companions whom the British Admiralty found increasingly necessary to keep its ships' captains sane on gruellingly long voyages while maintaining a strict social distance from the rest of the crew, as laid down by regulations. Darwin's less affluent contemporaries T. H. Huxley and Joseph Hooker had to make do instead with service as ships' surgeons, the duties of which Huxley in particular was to find a continually frustrating distraction.

The best solution was something between those extremes: funded and sponsored by government, which helped to open official doors, but travelling on alone, which maximized freedom of action. Such was the dual privilege enjoyed by Hooker for his classic journeys to the Himalayas which are best remembered today as the origin of so many of our garden rhododendrons. Yet even that loftiness of status was not enough to save Hooker from much time-wasting obstructiveness and, on one occasion, even risk to his life whenever he ventured outside regions under British jurisdiction.

Most of Raby's subjects were male (and most, too, were British), but he does not overlook the indomitable Mary Kingsley, who foraged among the mangrove swamps of West Africa without forgoing her normal full skirts, and armed with nothing more fearsome than an umbrella. Nor does he fail to include the imperious Marianne North, who captured in paint the jungle flora that her predecessors had thought only to collect.

A specialist in English literature, Raby devotes a closing coda to examining how the Victorian scientific travellers changed the European view of the world and led to the rise of a new mythology mediated through the novels of writers such as Rider Haggard and Joseph Conrad. To provide a sense of time and place, the book is illustrated with well-chosen portraits of some of the chief figures discussed, as well as with a series of maps of tropical regions taken from *The Times Atlas* of 1895; these last, alas, are sadly muddy. □

David E. Allen is at the Wellcome Institute for the History of Medicine, 183 Euston Road, London NW1 2BE, UK.



WHILE travelling abroad, beware of the mantte, or manta ray, which was thought to be very fond of pearl-divers. This is one of about 200 watercolours painted by French Huguenots accompanying Sir Francis Drake on his voyages to the West Indies. The collection, known as *Histoire naturelle des Indes* and held at the Pierpont Morgan Library, New York, has been reproduced in full in *The Drake Manuscript* (André Deutsch, £39.95).

Cycling through Arabic history

Ziauddin Sardar

Encyclopedia of the History of Arabic Science. Vol. 1: Astronomy — Theoretical and Applied. Vol. 2: Mathematics and the Physical Sciences. Vol. 3: Technology, Alchemy and Life Sciences. Edited by Roshdi Rashed. *Routledge*: 1996. Pp. 1,105. £160, \$250.

THE study of the history of Islamic science can be charted through at least four cycles. During the eighteenth century, when history of science emerged as a discipline, it occupied a privileged position. For the Enlightenment philosophers and historians of science, as Roshdi Rashed tells us, the development of Islamic science provided the guarantee of continued progress during a period dominated by superstition and darkness.

Colonialism marked the beginning of the second cycle. The colonial outlook, combined with German Romantic philosophy and philology, consigned the importance of Islamic science to the outer limits. *De jure*, Rashed writes, Islamic science lost its right to exist, while *de facto* it was indispensable to historians, who increasingly referred to it.

The third cycle began in the 1950s when George Sarton's monumental *Introduction to the History of Science* once again placed Islamic science on the scholarly map. As a result, a whole generation of scholars came to the field with a new determination to discover the true scope and magnitude of Islamic science in history. *The Encyclopedia of the History of Arabic Science* marks the beginning of the end of this cycle. It brings together the scholars who pioneered the field during the 1960s, 1970s and 1980s and tries to consolidate their new knowledge.

It is not an encyclopaedia in the conventional sense of containing alphabetical entries. Nor is it encyclopaedic in range: it has serious disciplinary gaps and by no means reduces all we know about Islamic science to a form accessible to students and the educated general public. Rashed claims that it is the first synthesis in this area. It does indeed provide us with a synthesis, but not a total synthesis of what we have learned about the history of Islamic science and its various subdisciplines over the past decades. What we are offered is a synthesis by each author of his own work based on previously published papers in his particular field. This kind of synthesis has recently become common as the third-cycle generation reaches retirement age: many anthologies by historians such as E. S. Kennedy, David King, A. I. Sabra have appeared recently.

Third-cycle contemporaries have two main characteristics. By and large, they are positivist and believe that science is value-free and international. They attempt to show the universal character of Islamic science, seeking to present it as a secular enterprise and shunning any analysis of the context of religion and civilization in which it evolved and developed. Furthermore, they are concerned more with textual analysis — that is, discovering, editing and translating manuscripts — than with interpretative history or historical theory-building. The overall emphasis is on 'hard' facts and discovering the 'real' content of Islamic science.

The encyclopaedia contains all the hallmarks of this generation of scholars. It

Jolivet omits two of the most noteworthy classifications of all: the overtly religious classification of knowledge by al-Ghazzali and the more general one by ibn Khuldun. The former has been blamed by many scholars for the decline of science in Muslim civilization; the latter tried to limit the damage.

The chapter on algebra by Rashed ignores the fact that al-Khwarizmi's book was a result of a religious impulse: he was, above all, trying to develop a system for the equitable distribution of inheritance as dictated by Islamic law. Similarly, other chapters talk about developments in various disciplines as though they were totally divorced from society and culture.

The encyclopaedia also lacks an overall epistemological or philosophical framework to put the discoveries into perspective. One of the most vibrant characteristics of classical Muslim civilization is the heated discussion it generated on theories of knowledge. And philosophy of science was a contentious and contested territory for Muslim scholars and scientists. As Muhsin Mahdi writes in a reflective chapter, included as an afterthought in the third volume, "Arabic science and philosophy cannot be separated in the period under discussion without doing violence to each of them". By finding no place for chapters on epistemology and philosophy of science, the encyclopaedia does just that.

There is another bias worth mentioning. The contributors are drawn largely from a group of ageing Arab nationalists and French orientalist scholars (five of whom died while the work was with the publisher). This group, led by Rashed, is antagonistic — for historical reasons too complicated to explain here and concerned mainly with personal egos — with the other notable group of ageing historians of Islamic science led by A. I. Sabra of Harvard University. Many senior scholars from Sabra's group, as well as many other important workers such as Franz Rosenthal, Albert Iskandar, David Pingree and Faut Sezgin, are conspicuous by their absence. The encyclopaedia therefore suffers from the limitations and the biases of one particular group of historians. Hence the use of the term "Arabic science" — based on the argument that it was the common language in which science in the Muslim civilization was written — rather than the more common term "Islamic science", and the emphasis on mathematical sciences, which is the main strength of this group, at the expense of life sciences.

But if one is willing to overlook its pretensions to be an encyclopaedia, one will find in this book a good collection of papers. Rashed does succeed in raising the partial, secular vision of third-cycle scholars into a higher and broader whole. The first and second volumes in particular provide us with fairly extensive and original surveys on astronomy, mathematics,

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A physician taking the pulse of a patient.
From the front binding board of the *Canon of Medicine* by Avicenna (AD 980–1037).

largely examines the 'exact sciences', with the contributors piling up fact after dry fact, making most chapters difficult to read. Apart from King's chapter on astronomy, none of the contributors places the discoveries of Muslim scientists in appropriate contexts of religion and civilization.

Rashed does pay lip-service to the importance of seeing Islamic science in its proper setting. For a satisfactory knowledge of Arabic science, he says, "it is necessary to restore it to its context, to the society which witnessed its birth... how indeed can one understand certain aspects of its development if one forgets the Islamic city and its institutions?". How indeed? Yet the contributors not only forget the context, but, it seems to me, they also go out of their way to expunge the religious and civilization basis of Islamic science. For example, the appalling chapter on the classification of the sciences by Jean

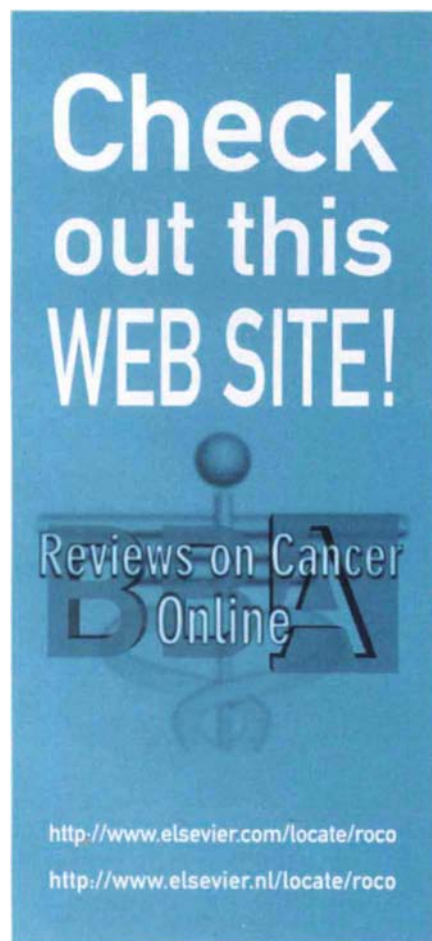
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mathematical geography and optics, although the third volume is rather pedestrian.

We are now entering the fourth cycle in the evolution of the history of Islamic science as a fully fledged discipline. A host of younger scholars such as Syed Nomanul Haq, Ahmad Dallal and Jamil Raghip, who focus simultaneously on context and content, are beginning to make their mark. It is left to them to produce a genuine encyclopaedia on Islamic science: one that will not only collect everything that is known in the field but also place it in its context of religion and civilization — an integrated synthesis that will make the history of Islamic science truly accessible to beginners and students. Above all, it should be an encyclopaedia that rises above petty quarrels between ageing scholarly groups and embraces the expertise and perspectives of all scholarly endeavours in the field. □

Ziauddin Sardar, a consulting editor of *Futures* and visiting professor of science and technology policy at Middlesex University, is at 1 Orchard Gate, London NW9 6HE, UK.

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Double identity

John Galloway

Two or The Book of Twins & Doubles. By Penelope Farmer. *Virago: 1996. Pp. 482. £20.*

THE idea of twins, particularly identical twins, is too good to have been missed by nature or by art. Science is littered with attempts to use twins to solve genetic puzzles, such as diabetes, by exploiting the opportunities offered by two people with exactly the same genes. Mistaken identity, an enduring theme in literature and the cinema, relies heavily on the idea of identical twins indistinguishable even to those closest to them. Mythology is crammed with twin deities, often moral (or other) opposites, who embody the struggle between good and evil, or life and death; these ideas are also common in films such as *The Black Room* and *The Dark Mirror*.

And what about life itself? In societies where the pressure to conform is in constant conflict with the ideal of individual expression, it is hardly surprising that twins arouse so much interest and are the subject of so much misunderstanding. There is a marked polarity between parents who emphasize the sameness of their twins and those who try to disguise and counter it.

But the idea of twins extends beyond people into the physical sciences, and there are twin metaphors in the complementary strands of DNA and the mutual mirror images of enantiomeric molecules.

Penelope Farmer is herself a twin and a writer in her own right. She wrote this book after the death of her twin sister as a way of trying to understand through the minds of others what being a twin means. The "others" are a rich and varied crowd. I was not surprised to find Carl Jung included, but finding the footballer Eric Cantona was unexpected. Mary Kingsley, the nineteenth-century traveller and writer, is startlingly direct and disturbing in *Travels in West Africa*: "Twins are killed among all the Niger Delta tribes and in districts out of English control, the mother too is killed." This piece represents one extreme of society's reaction to multiple births. It is one solution to the problem forced on Olivia Dionne, father of the famous quintuplets; he fled from the room when they were born in a state of nervous collapse, crying "what am I going to do with five babies?". However, their local doctor solved the problem, at least

for himself and the rest of the town. The parents' views were not sought.

The book is an anthology of writings about twins, both fact and fiction, but is not, as the blurb claims, exhaustive; it also lacks a proper bibliography. It falls down too in its rather thin coverage of modern popular fiction. John Barth gets a couple of quotations, but there is nothing from his cult classic *The Sot-Weed Factor*, whose hero, Ebenezer, the "Poet Laureate" of Maryland, is a twin. Louis de Bernière's *The Troublesome Offspring of Cardinal Guzman* is also well worth a reference. The "Mexican musicologist" hero meets a girl called Ena, makes love to her and discovers she is a virgin. The next night he makes love to her again,

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Double vision? Arnold Schwarzenegger and Danny DeVito look less than identical in the film *Twins*.

only to discover that she is still a virgin! Work that one out.

It is perhaps only to be expected that the most forceful writing is about how twins view each other rather than how other people view twins. Real twins do it better than the twins of imagination. There are many extracts from Marjorie Wallace's *The Silent Twins*, an account of the lives of the identical twins June and Jennifer Gibbons. Highly literate and articulate, for most of their lives Jennifer and June communicated only with each other. After an arson attack, they were detained in Broadmoor, a maximum-security hospital for the mentally ill who have committed serious criminal offences. Jennifer died of myocarditis on the day they were released. June's reaction was one of exultation at being freed from the exclusive relationship, mixed

with deep grief and bitter anger that her special day had been ruined. In some ways it was a sweet release: "We were war weary. It had been a long battle. Someone had to break the vicious circle." A month after Jennifer's funeral, June asked Wallace whether a banner could be floated across the skies of Haverfordwest. When asked what it would say, she replied: "June is alive and well and has at last come into her own."

The vividness and power of these extracts makes you wonder why there are not more contemporary examples of twins talking about themselves. The book could perhaps have quoted from *How Twins Grow Up* by Mary Rosambeau; not literary maybe, but certainly real and immediate.

Farmer's book is a rich and strange slice of the literary cake. Twins themselves and ideas about twins are seen from every perspective and by every kind of writer: from St Augustine to Kurt Vonnegut; from Galton to Graves; from Shelley to Poe. There are twins as variations on the same person; twins as two different people; twins in myths, and myths about twins; and twins as the pivot on which every kind of literary plot turns. For most people, perhaps there is more about twins than they want to know. For those of us who have twins of our own, it's great. □

John Galloway is at the Eastman Dental Institute, 256 Gray's Inn Road, London WC1X 8LD, UK.

Brown fables, green wit

Stuart Pimm

Betrayal of Science and Reason. By Paul R. Ehrlich and Anne H. Ehrlich. *Island Press*:1996. Pp. 320. \$24.95.

THE Ehrlichs have been insulted, their sanity questioned, their writings and depressingly accurate apocalyptic predictions misquoted, their humanity denied, and their friends likewise besmirched. Like the Ehrlichs themselves, this is a wickedly funny book and it takes no prisoners among their critics. Panglossian critics of the Ehrlichs are skewered with their own words and simple, choice responses. For good measure, the book addresses why so much of the scientific opinion is so readily represented to the public as "the hysterics of a few pseudo-scientists".

The core chapters are about fables. Paul Ehrlich attained notoriety with *The Population Bomb* (Amereon, New York), so fables of food and famine, disease and

death appropriately come first. Julian Simon claims we have "the technology to feed an ever-growing population for the next 7 billion years"; Malcolm Forbes that population growth in the United States "is not a problem because it's still nowhere near as dense as the Netherlands"; Dixy Lee Ray that the human population has not exploded; and others claim that medicine has eliminated infectious diseases, and famines are now only history. The pithy response to Simon is that if the population grew at only a millionth of its current rate, its mass would exceed that of the Universe well before the allotted time; presumably, the population would have to travel outwards faster than the speed of light to occupy the space.

Our population has expanded with clockwork precision from 3.5 billion when Ehrlich wrote his 1968 bestseller to almost exactly the 5.65 billion he predicted then for 1995. Assertions of the elimination of infectious diseases will hardly comfort the millions of children who die annually of gastro-intestinal infections, or adults who are now HIV-positive. "Emergent diseases" emerge, of course, because a disease may spread through a dense population but fizzle in a sparse one. Now, more than ever, we are abundant, concentrated in cities, and travel widely and rapidly, spreading diseases more efficiently.

What of the famous prediction that "hundreds of millions of people are going to starve to death... in the 1970s"? The Ehrlichs are delighted to report that they were wrong. Global programmes to improve food distribution reduced the decade's total deaths to between 100 million and 140 million, plus another 100 million since. Looking at these numbers, one wonders what authors like Michael Fumento (*Science Under Siege*; William Morrow, New York) could have been thinking in asserting that "none of [the Ehrlichs'] predictions ever come true", and that they were "off, by hundreds of millions".

The future will be much better, of course: *The Economist* (12 August 1995) tells us so. "Unimagined scientific breakthroughs will boost crop yields." The Ehrlichs ponder why this respected publication had not posited equally "unimagined breakthroughs (that) will allow a runner to run a two-minute mile". With more people, there will be more geniuses to solve our problems. Surprisingly, they seem to be in short supply in the former Yugoslavia and central Africa, where people are numerous, productive land scarce, and the problems deadly.

Fables about physical resources, economics, toxic substances and species extinction follow. English readers of *Nature* will be reassured that exposure to dioxin is no more dangerous than the

week of sun and sand to which they look forward in the dark, damp days of February. Just don't count on global warming to improve the weather. Another fable; how could it be otherwise? As Gregg Easterbrook asks in *A Moment on the Earth* (Viking, New York), "since CO₂ is only 1% of the atmosphere, why should we care if its concentration doubles?"

The ozone myths are more interesting. Volcanoes spew out large amounts of chlorine, and whether they or chlorofluorocarbons (CFCs) are the principal cause of the ozone hole is a legitimate scientific question. As it turns out, volcanic chlorine is mostly HCl, and so is soluble in rain, whereas CFCs are insoluble and reach the stratosphere. This distinction between chlorine compounds is lost on the American talk-show host Rush Limbaugh, who continues to deny mankind's culpability. One can only hope that he learns to distinguish NaCl from HCl, lest his millions of loyal fans lose their only source of science news.

The Ehrlichs stress how extensive is excellent reporting of science and its debates. Capable reporters do not feel compelled, for the sake of 'balance', to report the views of flat-earthers every time NASA publishes a photograph of Earth from space. Few have been in the glare of the media for as long as the Ehrlichs, whose recommendations on how to work with the media are short and useful. Those who can, should, presenting their conclusions first, avoiding stories of future scenarios, and carefully separating what actions are ideal and what are practical.

To distinguish good and bad science reporting, there is a simple field-guide quality to writing that one can easily pass on to students. Is the publication adequately referenced, or are there large tracts of completely unsupported statements? Is it peer reviewed? Are the peers few or numerous? Are they truly peers? Where do they publish? Like being a world-class cricketer or concert pianist, it's hard to fake being a world-class scientist. These questions reveal all but the rare, well-qualified contrarians. To evaluate their claims, are there international, consensus statements, as there are on population, biodiversity and climate change?

If some politicians cannot recognize these obvious distinctions, the good news is that some can, and they act accordingly. It is to two of these, the late Republican senator John Heinz and his wife, that the Ehrlichs dedicate their book. □

Stuart L. Pimm is in the Department of Ecology and Evolutionary Biology, University of Tennessee, Knoxville, Tennessee 37996, USA.

A massive phytoplankton bloom induced by an ecosystem-scale iron fertilization experiment in the equatorial Pacific Ocean

Kenneth H. Coale^{*}, Kenneth S. Johnson^{*†}, Steve E. Fitzwater^{*},
R. Michael Gordon^{*}, Sara Tanner^{*}, Francisco P. Chavez[†], Laurie Ferioli^{*†},
Carole Sakamoto[†], Paul Rogers[†], Frank Millero[‡], Paul Steinberg[‡],
Phil Nightingale^{§||}, David Cooper^{§||}, William P. Cochlan^{||}, Michael R. Landry[#],
John Constantinou[#], Gretchen Rollwagen[#], Armando Trasvina^{☆||}
& Raphael Kudela^{||}

^{*} Moss Landing Marine Laboratories, PO Box 450, Moss Landing, California 95039-0450, USA

[†] Monterey Bay Aquarium Research Institute, PO Box 628, Moss Landing, California 95039-0628, USA

[‡] University of Miami, 4600 Rickenbacker Causeway, Key Biscayne, Florida 33149, USA

[§] School of Environmental Sciences, University of East Anglia, Norwich NR4 7TJ, UK

^{||} Hancock Institute for Marine Studies, University of Southern California, Los Angeles, California 90089-0371, USA

[#] Department of Oceanography, University of Hawaii at Manoa, 1000 Pope Road, Honolulu, Hawaii 96822, USA

[☆] CICESE Oceanografía Física, Km 107 Carret. Tijuana-Ensenada, Ensenada, B. C. Mexico

The seeding of an expanse of surface waters in the equatorial Pacific Ocean with low concentrations of dissolved iron triggered a massive phytoplankton bloom which consumed large quantities of carbon dioxide and nitrate that these microscopic plants cannot fully utilize under natural conditions. These and other observations provide unequivocal support for the hypothesis that phytoplankton growth in this oceanic region is limited by iron bioavailability.

THE persistence of high-nitrate, low-chlorophyll (HNLC) conditions in the surface waters of several large regions of the world's oceans comprise a familiar enigma in oceanography¹. The factors that prevent the utilization of nitrate also regulate the rate at which carbon dioxide is taken up by phytoplankton and, ultimately, the amount of carbon exported from the surface waters. The oceans are both a major source and sink for atmospheric carbon dioxide, and processes that control the balance of these fluxes are thought to have a major effect on global climate². Understanding the factors that limit the uptake of excess plant nutrients is, therefore, a key to understanding climate change. Grazing pressure exerted on phytoplankton by rapidly reproducing microzooplankton and micronutrient (iron) deficiency may function jointly in these HNLC waters³; yet the relative importance of each of these factors in controlling the biomass and rates of phytoplankton production has remained contentious⁴. The experimental tools available to the oceanographer have, until recently, been inadequate to resolve the relative importance of these processes. *In vitro* enrichment experiments^{5–12}, where iron is added at nanomolar levels to samples of sea water, invariably do not represent the *in situ* phytoplankton grazer community. The interpretation of such experiments has, therefore, been ambiguous to some^{13–15}.

In 1993, the first open-ocean iron enrichment experiment

(IronEx I) was performed in an attempt to eliminate the ambiguity of *in vitro* containment and allow the processes influencing community structure and carbon export to operate at an appropriate scale¹⁶. IronEx I involved a single 4 nM enrichment of dissolved iron to an experimental 'patch' of equatorial Pacific surface waters¹⁶. This experiment demonstrated a clear and unambiguous physiological response to the addition of iron which resulted in a doubling of plant biomass, a tripling of chlorophyll concentrations and a fourfold increase in phytoplankton productivity. Four days after the iron addition, the patch was subducted beneath a layer of less-dense surface water; hence, the magnitude of the biological and geochemical response was much smaller than predicted from previous bottle enrichment experiments^{5–12}. Nitrate drawdown during IronEx I was undetectable ($<0.2 \mu\text{M}$), and carbon dioxide fugacity was only reduced by $10 \mu\text{atm}$ (ref. 17).

Several hypotheses were advanced to explain this small biogeochemical response. (1) Iron was rapidly lost from the patch¹⁶, (2) the subduction of the patch to lower light levels minimized the photodissolution of iron colloids and decreased rates of bioavailable iron production¹⁸, (3) zooplankton quickly cropped the increase in phytoplankton biomass^{16,19}, and (4) another nutrient, such as zinc or silicate, became limiting thus preventing further growth^{16,20}. The second mesoscale iron enrichment experiment (IronEx II) was designed to test these hypotheses. Multiple additions of iron, over several days, were used to simulate a natural iron input event, and zooplankton grazing rates and concentrations of other potentially limiting nutrients were closely monitored over the course of the experiment. The massive phytoplankton bloom triggered by the relief of iron-limitation was not significantly checked by either grazing or secondary nutrient limitation, thus unequivocally supporting the hypothesis

^{||} Present addresses: Plymouth Marine Laboratory, Prospect Place, West Hoe, Plymouth PL1 3DH, UK (P.N.); Department of Atmospheric and Oceanic Sciences, University of Wisconsin, 1225, West Dayton Street, Madison, Wisconsin 53706, USA (D.C.); Monterey Bay Aquarium Research Institute, PO Box 628, Moss Landing, California 95039-0628, USA (R.K.); Departamento de Oceanografía Física, Oceanografía Tropical, CICESE subsele Baja California Sur, Miraflores No. 334 entre Mulege y La Paz, Fracc. Bella Vista, La Paz 23050, B.C.S., Mexico (A.T.).

that the HNLC condition of these waters is due to iron-limitation of algal growth.

Experimental strategy

The IronEx II experiment involved three separate mesoscale infusions (patches 1, 2 and 3) and a series of deckboard *in vitro* enrichment experiments. A 1,000 km² area was surveyed in the vicinity of 3.5° S, 104° W in May 1995 to ensure that hydrographic, biological and chemical conditions were uniform throughout the region, to minimize the chance of surface-water subduction which biased the first iron enrichment experiment, and to ensure that changes in the experimental area could be attributed to the presence of iron.

Many of the experimental methodologies originally developed during IronEx I¹⁶ were employed in the IronEx II experiment. All navigation was conducted relative to a central buoy, fixed within the mixed layer of the patch using a 1 × 20 m holey-sock drogue, that was set at a depth of 15 m. This buoy, instrumented with a global positioning system, packet radio, fluorometer, transmissometer, conductivity, oxygen and temperature sensors, served as the centre of a lagrangian frame of reference. The position of the buoy was transmitted to the ship every 5 min. Iron infusions and sampling transects were performed relative to this lagrangian buoy. The buoy drifted at about 2.8 km h⁻¹ in a south-by-south-westerly direction over the duration of the experiment. A second instrumented buoy was deployed outside the iron-enriched area to monitor changes in the unperturbed waters. The central buoy was recovered 19 days after deployment, some 1,500 km from its initial position (Fig. 1).

On 29 May (day 0 of the patch 1 experiment), 225 kg of iron (as acidic iron sulphate) was mixed in constant ratio with the inert chemical tracer sulphur hexafluoride (SF₆) and then injected immediately aft of the ship's twin propellers as the ship steamed over a 72 km² rectangular deployment grid. Acidic iron sulphate is the form of iron thought most likely to enter the surface waters

naturally through atmospheric deposition²¹. Based on a turbulent dispersion model²², the iron streaks laid 400 m apart in the ship's track were calculated to merge within one day, resulting in a uniform addition of iron throughout the 25-m mixed layer. This uniformity was confirmed by both underway measurements and vertical profiles of SF₆ and Fe. Mixed-layer depth averaged 25 m during the infusion, and the initial (day 1) concentration was 2 nM Fe. Two subsequent infusions each of 112 kg of Fe (to yield an increase of 1 nM each) were performed on days 3 and 7 of the experiment to maintain an enhanced concentration of iron in patch 1. All infusions were performed using even track spacing. Subsequent surveys were both rectilinear and star-shaped.

A suite of hydrographic and biological measurements (temperature, salinity, Fe, SF₆, CO₂, dimethyl sulphide and its algal precursor, nutrients, primary productivity, trace elements, plant pigments including chlorophyll, grazing pressure, phytoplankton, zooplankton and heterotrophic bacterial abundances, isotopes (¹⁶O, ¹⁸O, ¹²C, ¹³C, ²³⁴Th), colloids and metal speciation) were performed each day at vertical profile stations both inside and outside the experimental patch. Many of these data we report here, and in companion papers in this issue, and others will be reported elsewhere. 'Inside' stations were defined both by their

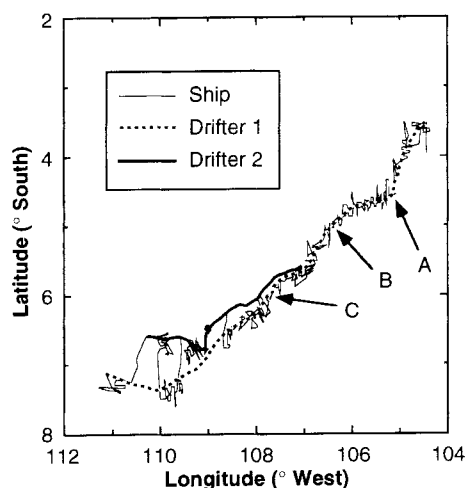
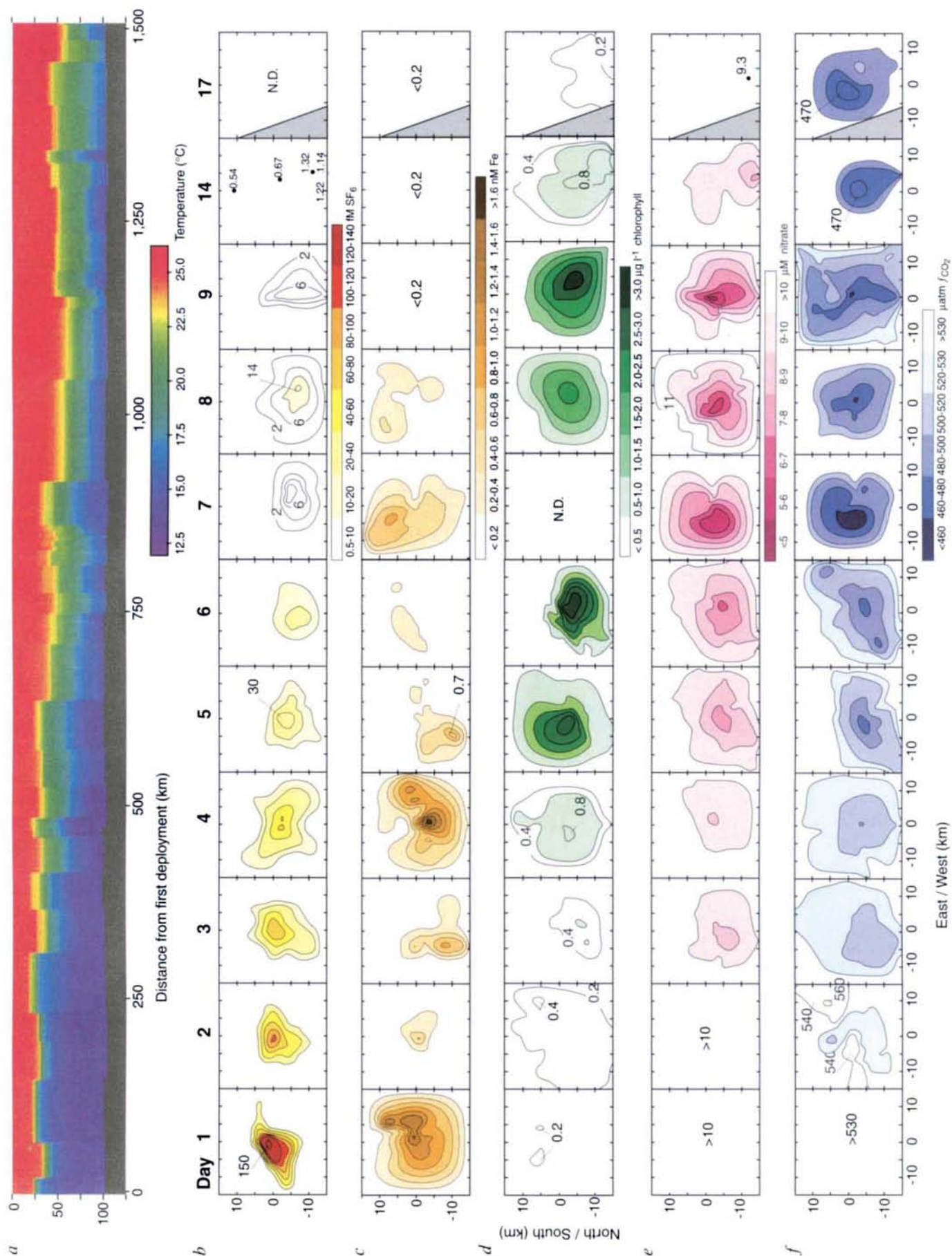


FIG. 1 The drift tracks of the lagrangian drifter buoys, indicating the centre of the enriched area, are plotted together with the ship's position during the IronEx II experiment. The dashed line indicates the track of the central lagrangian drifter buoy (drifter 1) marking the centre of patch 1 (sequentially infused). The thick solid line indicates the track of the central lagrangian drifter buoy (drifter 2) marking the midpoint between patch 2 (control; no iron addition) and patch 3 (+0.3 nM Fe). The thin line indicates the ship's track as it navigated about both buoys during the 19 days of the experiment. Initial patch 1 advection velocity (as determined by buoy 1) was close to 3 km h⁻¹ requiring constant corrections to the lagrangian coordinate system. Buoy positions were transmitted to the ship every 5 min and were used to continuously update the navigational frame of reference. Arrows A, B and C designate the location of the initial 2 nM, and subsequent 1 nM, Fe infusions of patch 1, respectively. The drift track of the outside buoy is not shown.

FIG. 2 A series of contours of six measured variables plotted as a daily time series over the duration of the patch 1 experiment. These plots were constructed using Systat (statistical software from Systat, Inc., Evanston, IL, USA) with the data available from vertical profiles, underway sensors and discrete mixed-layer samples. The underway data was obtained using the ship's PVC pumping system which had an intake on the bow, ~6 m below the surface. On day 17, at the southwesternmost extent of the patch 1 experiment, a water mass of lower density and lower nutrients was encountered. The approximate location of this abutting water mass is depicted as a grey area in plots b-f on day 17. a, Vertical section of temperature along the drift track of buoy 1 (ordinate, depth in metres). This figure was constructed from vertical temperature profiles taken in or near patch 1 using a profiling conductivity-temperature-depth sensor deployed from the ship. It is constructed from discrete casts taken at various time intervals throughout the experiments and, therefore, does not represent a continuous trend in the temperature structure. Nonetheless, the general features of the thermocline can be seen from this plot. b, Areal contours of SF₆ concentrations in patch 1 over the course of the experiment. SF₆ was used as an inert chemical tracer marking the infused area and provided indication of the enriched waters after iron had been removed. Initial concentrations decreased rapidly due to outgassing to the atmosphere and mixing both vertically and horizontally. Tracer mixed below the thermocline was lost due to differential advection of the surface layer. Measurements of SF₆ were made using detection methodology described previously¹⁵. The gaps in the data on days 10-13, 15-16 represent times when the ship was at patch 2 and 3. The N.D. (not determined) for day 17 indicates that no underway determinations were made that day. c, Daily areal plots of iron concentration contoured over the course of the experiment. Underway measurements of iron were made using flow injection analysis with chemiluminescence detection⁴⁴ further modified for analysis at sea. Owing to the contamination near the ship, the ship's pumping system and the short sample load-times required for high spatial resolution mapping, the detection limit of the combined system was ~0.2 nM. Background values of ~0.02 nM dissolved iron were confirmed by electrochemical (E. Rue, personal communication) and modified atomic absorption methods⁴⁵. These concentrations were encountered outside patch 1 and within patch 1 after day 9. d, Chlorophyll contour plots were generated using a flow-through fluorometer and calibrated with filtered chlorophyll samples extracted in acetone for 24 h at -20 °C and read on a calibrated fluorometer. The N.D. on day 7 indicates that no determinations were performed through the ship's pumping system on this day. e, Nitrate contours were constructed from measurements made while underway from the ship's flow-through system using an Alpkem segmented flow auto-analyser. The continuous nitrate record was calibrated with periodic standards and a daily series of discrete samples and standards run on an independent analyser. f, Areal contours of CO₂ fugacity (*f*_{CO₂}) were determined using a flowing system in which the water at 6-m depth, and air-equilibrated samples were measured with a LICOR non-dispersive infrared detector. The accuracy of the system was determined during the cruise with two standard gases (277.05 and 501.73 p.p.m. standards) that were measured every two hours.



proximity to the central buoy and by the SF_6 concentration. 'Outside' stations were located at areas with background levels of SF_6 and were generally near the outside buoy. Underway surveys using the ship's pumping system were conducted daily between inside and outside stations. The trends described here are based on measurements taken within the mixed layer at the daily inside stations. The seawater intake used for underway measurements was located on the bow at a depth of 6 m and therefore represents the water of the upper mixed layer.

Two smaller (24 km²) patches were also created. One (patch 2) was infused with acidified sea water and SF_6 , but no iron. This patch served as a control to test for possible effects due to transient acidification and the presence of the research vessel. The other (patch 3) received a single, low-level addition of iron (to 0.3 nM) plus SF_6 . Patch 3 was created to mimic the concentration of iron associated with the equatorial undercurrent which upwells into surface-waters to the west of the study area²³.

Patch behaviour

Patch coherence was essential to the success of the experiment. The mixed layer deepened in a series of small mixing events from 25 m on day 1 to about 50 m by day 11 (Fig. 2a). As the mixed layer deepened, patch 1 mixed with higher-nutrient waters directly below. This was evident in periodic increases in nitrate concentrations within patch 1 and a deepening of the thermocline and SF_6 signal over time. Contours of SF_6 concentrations measured in the surface waters over the course of the experiment (Fig. 2b) indicated that patch 1 was fairly cohesive and expanded with time from an initial area of approximately 72 km² to over 120 km² by day 17. As the residence time of iron in these waters was short (see below) and the buoy slipped somewhat relative to the mixed layer owing to wind drift, SF_6 was used as the primary indicator to track the area enriched with iron and to distinguish 'inside' from 'outside' stations.

Rapid uptake/removal of the iron was observed following each of the iron infusions on days 0, 3 and 7 (Fig. 2c). As the biomass of patch 1 increased in response to added iron, the rate of iron removal also increased. Discrete iron analyses of mixed-layer samples indicated that iron concentrations decreased to below ambient levels (<0.02 nM) within three days following the last iron addition, (E. Rue and R.M.G., unpublished data) (Fig. 2c).

Biological and geochemical responses

Fluorescence measurements in patch 1 showed a rapid and monotonic increase in chlorophyll-*a* concentrations from an initial value of 0.15–0.20 $\mu\text{g l}^{-1}$ to values approaching 4 $\mu\text{g l}^{-1}$ on day 9, two days after the last infusion of iron (day 7). Maximum chlorophyll-*a* concentrations in patch 1 were 27 times the mean of initial and outside chlorophyll-*a* concentrations. Chlorophyll-*a* concentrations then decreased throughout the rest of the experiment, reaching 0.30 $\mu\text{g l}^{-1}$ on day 17 (Fig. 2d). Increased chlorophyll concentrations were found throughout the mixed layer in the patch (Fig. 3b).

The phytoplankton chlorophyll in patch 3 (+0.3 nM Fe) also showed a distinctive response to iron, increasing from 0.22 to 0.44 $\mu\text{g l}^{-1}$ after two days. The response of patch 3 is consistent with incubation experiments indicating a Michaelis–Menten relationship between dissolved iron concentration and community growth rates²³. Given a half saturation constant of 0.12 nM (ref. 23), the addition of 0.3 nM iron should produce a two-fold change in community growth rate. The threshold for community response to iron addition is clearly at the sub-nanomolar level. No biological response was detected in the control patch which received only acidified sea water and the inert tracer SF_6 . From this, we conclude that the observed community responses in patches 1 and 3 were due to the iron added and not to other chemicals or the presence of the ship. The remainder of this discussion will focus on the results of patch 1.

Nutrients were measured both continuously through the ship's seawater system, and in all discrete samples. Contour plots of

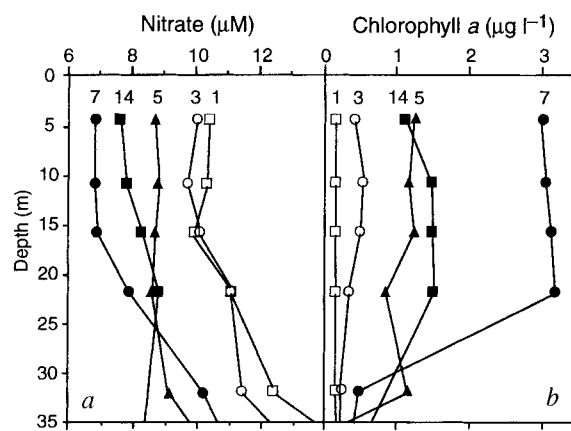


FIG. 3 a, Vertical profiles of mixed-layer nitrate from the daily 'inside-patch' stations of patch 1. Numbers at the top of each profile indicate the day of the patch 1 experiment. These plots illustrate the depletion of nitrate as the bloom reached its peak near days 7–9. The subsequent increase (day 14) is thought to be the result of mixing. Nitrate concentrations both inside and outside the patch converged to about 10 μM by ~50 m. b, As a but for mixed-layer chlorophyll *a*.

nitrate concentrations in surface waters throughout the experiment (Fig. 2e), as well as nitrate profiles at inside-patch stations (Fig. 3a), indicated a strong drawdown of approximately 5 μM nitrate as the biological response developed. Some nitrate may have been added by mixing from below as the mixed layer deepened (days 11, 14; Fig. 2a). Following an initial lag of one–two days, nitrate drawdown tracked silicate drawdown on an equimolar basis, suggesting that diatom growth was responsible for most of the nitrate uptake.

Rates of nitrate uptake by the planktonic assemblages were determined using the ¹⁵N-isotope tracer technique²⁴. Samples were collected using trace-metal-clean techniques²⁵ inside and outside (controls) of patch 1 from 15 m depth (~40% of the photon flux at 0.5 m) and incubated for ~6 h in Plexiglass incubators under simulated *in situ* light and temperature conditions. Absolute (transport) uptake rates of nitrate increased dramatically (14-fold increase) as a result of Fe enrichment, from <10 nM h⁻¹ (range 8.7–9.6 nM h⁻¹ in control and pre-fertilization samples) to a maximum rate of 133 nM h⁻¹ on day 6. Addition of Fe also increased biomass-specific rates of NO_3^- uptake by a factor of 5–7 by days 6 and 8, before declining to pre-fertilization rates. However, there was no discernible increase in particulate-nitrogen-specific uptake rates for the first 4 days after iron enrichment. The subsequent increase in particulate-nitrogen-specific uptake at the height of the iron-induced bloom is the result of faster rates of NO_3^- consumption per unit phytoplankton biomass, a result similar to that reported for iron amended bottle experiments in the equatorial Pacific near 140° W (refs 3, 10). Post-incubation size-fractionation of ¹⁵ NO_3^- accumulation (from parallel filtrations on Whatman GF/F filters and Poretics 5.0 μm silver membrane filters) demonstrated clearly that the large phytoplankton (>5.0 μm size fraction) were responsible for the enhanced NO_3^- utilization caused by Fe enrichment. The uptake by the >5.0 μm size fraction, which accounted for <15% of the total uptake before fertilization, increased to 85–98% of the total NO_3^- uptake at the peak of the phytoplankton bloom (days 6 and 8).

The carbon dioxide fugacity (f_{CO_2}) calculated from continuous CO_2 measurements of CO_2 partial pressure (p_{CO_2}) on board the ship, showed significant depletions within patch 1 which paralleled the drawdown of nitrate (Fig. 2e). The maximum depletion in f_{CO_2} was observed on day 9, coincident with the maximum in most other biological and chemical indicators of growth. The south equatorial Pacific at 105° W is a strong source of CO_2 to the

atmosphere (f_{CO_2} in seawater, 526 p.p.m.; f_{CO_2} in the atmosphere, 360 p.p.m.) (Fig. 2f). Iron-enhanced growth resulted in a draw-down of about 90 μatm , which strongly reduced outgassing of CO_2 from these waters. In spite of this drawdown, levels of CO_2 were at or above atmospheric partial pressure, and we do not believe the system became carbon-limited. The lack of limitation by carbon (or other micronutrients such as zinc) was supported by bottle enrichment experiments which consumed all available nitrate (see below).

Community response

To examine the community response to iron addition, the taxonomic composition of the plankton was determined using epifluorescence microscopy. Taxa-specific cell volumes and densities, converted to carbon using known carbon:volume relationships^{26,27}, indicated that biomass increased in all phytoplankton groups (Fig. 4a,b). Diatoms clearly showed the greatest increase in biomass over ambient (85 $\times$). The smaller organisms were less affected, with *Synechococcus* (1 μm) and red fluorescing picoplankton (<2 μm) only doubling in biomass. The biomass of microzooplankton (<200 μm), primarily small ciliates and flagellates, increased in step with the smaller autotrophs (2 $\times$ increase). Mesozooplankton biomass (zooplankton >200 μm in length), comprised mainly of calanoid and cyclopoid copepods, also increased in patch 1 from 3.8 mg C m^{-3} measured from plankton net tows at control sites to 6.1 mg C m^{-3} in the patch. The highest mixed-layer concentration of mesozooplankton, 14 mg C m^{-3} , occurred in a daytime sampling of patch 1 on day 6.

Experimental dilution incubations²⁸ were performed to estimate both growth rates and grazing pressure on the small (<5 μm) phytoplankton that normally dominate the equatorial Pacific upwelling system²⁹⁻³¹ abundance. Phytoplankton abundance increased dramatically in patch 1 because they were able to grow faster, as a group, than the rates at which their consumers could remove them. Based on analyses of chlorophyll *a* in dilution incubations²⁸, the specific growth rate of the phytoplankton community averaged 1.25 d^{-1} , almost two cell divisions per day, during days 4-8. This was more than double the growth rate in ambient control waters, leaving a large imbalance between growth and

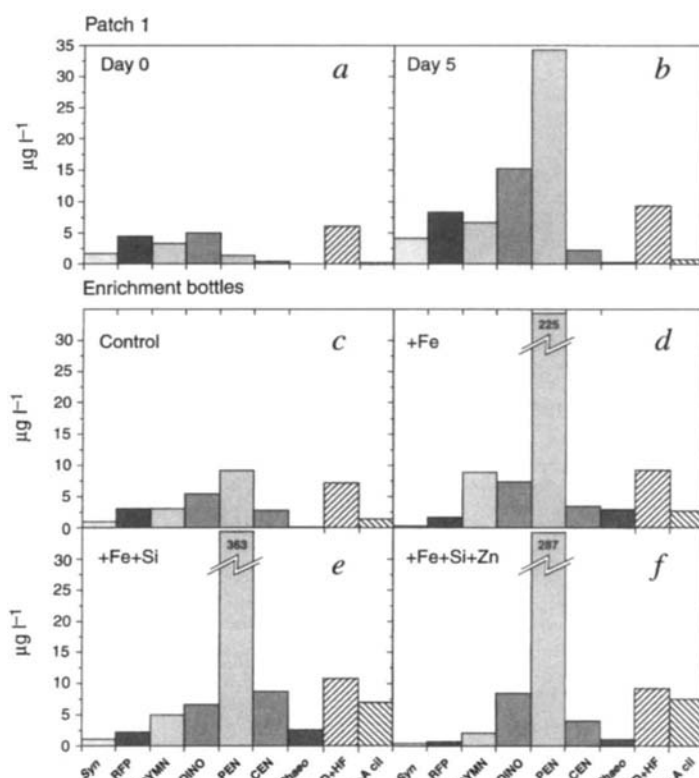
grazing processes in the early phase of the plankton bloom response to added iron. Microzooplankton grazing rate averaged 0.32 d^{-1} (range of 0.17–0.48 d^{-1}) at control sites out of the patch, but increased by more than three times in the patch to 1.1 d^{-1} on day 7, as chlorophyll was reaching its peak concentration. The modest increase in patch densities of picoplankton (for example, *Synechococcus* spp. and small fluorescing picoplankton) during the experiments indicates that the increase in microzooplankton grazing was almost sufficient to keep the smaller members of the phytoplankton community in check^{13,32}. In contrast, diatoms were clearly not contained by grazing, presumably because they were too large to be effectively consumed by the fast-growing microzooplankton, and too fast-growing to be suppressed by the larger mesozooplankton which have longer generation times with respect to the doubling rates of diatoms. This imbalance between production and grazing is analogous to the spring bloom of the North Atlantic³³.

The mesozooplankton grazing impact on phytoplankton, as deduced from analyses of gut pigment contents, was always small relative to that of the microzooplankton. The amount of phytoplankton pigment processed, per unit biomass of mesozooplankton, increased more or less in proportion to the increase in chlorophyll in patch 1. The community grazing effect of larger animals increased by about 50%, from 7.8% of phytoplankton standing stock per day at control sites to 11.4% d^{-1} in the patch, approximately in proportion to their increase in biomass. At the mixed-layer temperature of equatorial waters, the expected generation time of pelagic copepods of less than a week³⁴ could have allowed a more dramatic numerical response of the mesozooplankton to the bloom conditions. The lack of a stronger response could mean that these copepods are already growing at rapid rates in equatorial waters with high compensating rates of mortality due to predation.

Carbon removal

Measurements of both particulate and dissolved organic carbon (POC and DOC) concentrations throughout the experiments showed an increase in these two carbon pools. POC increased from 4 to 15 $\mu\text{M C}$ at day 6 in patch 1. The increase in living

FIG. 4 a, Plankton community composition within patch 1 at day 0 of the experiment as expressed in $\mu\text{g C l}^{-1}$. This composition is similar to that observed at the 'outside-patch' stations over time. The groups represented include: Syn, *Synechococcus* spp.; RFP, red fluorescing picoplankton; PRYMN, Prymnesiophytes; DINO, autotrophic dinoflagellates; PEN, pennate diatoms; Phaeo, *Phaeocystis*; HD + HF, heterotrophic dinoflagellates + heterotrophic flagellates; H + A cil, heterotrophic + autotrophic ciliates. Shaded bars indicate autotrophic biomass and diagonally hatched bars indicate heterotrophic biomass (the most likely grazers on the smaller size fraction of autotrophs). b, Taxonomic composition of patch 1 on day 5 of the experiment indicating increases in all classes of phytoplankton, especially the diatoms. c–f, Results of the bottle enrichment experiments performed on deck in 20-litre carboys⁸ to test the effects of other potentially limiting nutrients. Water was collected using 30-litre Go Flo bottles deployed on Kevlar hydrowire and tripped with a Teflon messenger. Water was transferred to acid-cleaned, 20-litre polycarbonate bottles within a class 100 clean lab, chained to the deck of the ship. Treatments include: c, control, nothing added; d, +2 nM iron added; e, +2 nM iron, +10 μM silicic acid; f, +2 nM iron, +10 μM silicic acid, +2 nM zinc. Results indicate that diatoms in bottle enrichments with added iron outperformed the mesoscale experiment and that bottles with added silicic acid enhanced diatom growth relative to those without silicic acid. Zinc did not appear to have a positive effect on growth. Note the scale break in the diatom bar. Numbers at the top of the bar indicate the micrograms of carbon per unit volume attained in this group.



biomass can account for about 75% of the increase in POC. The remainder can be attributed to the accumulation of detrital carbon as dead plankton remains, as there is no appreciable terrigenous source in this area. DOC also increased, and the change accounted for about 25% of the overall increase in fixed carbon. Overall, there was a net accumulation of approximately 14 $\mu\text{M C}$ in patch 1 on day 6. Oxygen concentrations within patch 1 show a 31 μM increase over initial stations. As exchange with the atmosphere would tend to reduce the oxygen anomaly, this value represents a minimum estimate of total new production during the experiment. Based on a Redfield carbon:oxygen ratio of 106:138, the corresponding estimate in terms of carbon is 24 $\mu\text{M C}$. Nitrate drawdown at this time was of the order of 4 μM , which corresponds to an organic carbon production of about 27 $\mu\text{M C}$. Similarly, the deficit in f_{CO_2} is equivalent to an increase in fixed organic carbon of 20 $\mu\text{M C}$.

The different estimates of carbon new production suggests that between 5 and 12 $\mu\text{M C}$ was exported from the surface layer. The lack of larger mesozooplankton grazers, which commonly produce rapidly sinking faecal pellets that transport carbon below the mixed layer³⁵, suggest grazing export did not remove this missing carbon. Much of the missing carbon lost from the patch was probably removed by vertical mixing and possibly sinking of diatom aggregates. There was a 20-fold reduction in SF_6 concentration, of which only 60% can be accounted for by exchange with the atmosphere. The remainder of the SF_6 , and much of the chemical and biological signal produced in the patch by iron enrichment, was eroded from the base of the mixed layer by exchange with waters moving relative to the advection of patch 1 and spread horizontally within the mixed layer.

Potential role of other nutrients

Shipboard bottle experiments (*in vitro* enrichments) were designed to test whether other factors such as zinc or silicate would reach such low concentrations that phytoplankton production would be limited after iron deficiency had been relieved. Zinc is required for both silicate uptake³⁶ and carbonic anhydrase activity, and it is depleted to subnanomolar concentrations in surface waters. Polycarbonate carboys (20-litre capacity) were filled with pre-enrichment water then augmented with the following treatments: [+0], [+2 nM Fe], [+2 nM Zn], [+10 $\mu\text{M Si}$], [+2 nM Fe, +2 nM Zn], [+2 nM Fe, +2 nM Zn, +10 $\mu\text{M Si}$]. The biological response in these *in vitro* experiments was monitored from sub-samples drawn from these carboys and analysed for a wide variety of parameters^{7,10}. The results from the addition of 2 nM Zn and 10 $\mu\text{M Si}$ (no added iron) were similar to the control (+0) and are not depicted here. The resultant taxonomic compositions are shown together with the patch 1 response in Fig. 4b–e. The results from the bottle experiments to which only iron was added were similar to those observed in patch 1. The treatments which included iron and silicate, both with and without zinc, may have slightly out-performed the treatment with iron but not containing silicic acid. This indicates that, following the relaxation of iron deficiency, supplemental amounts of silicate allow for greater diatom growth. There is some evidence to suggest that carbon uptake may be limited by carbonic anhydrase activity brought on by zinc deficiency³⁷ under bloom conditions where the concentration of CO_2 is low. Even at the picomolar concentrations of dissolved zinc detected (100 pM; R.M.G., unpublished results), our results (Fig. 4) are not consistent with zinc limitation in these waters, even when growth is stimulated by iron addition.

The species composition of the phytoplankton community that responds to iron addition can greatly influence the magnitude of the geochemical response. Our results indicate that iron enrichment favours diatoms. There are few natural instances where nitrate is in abundance and silicate is not. With few exceptions³⁸ therefore, silicate is not likely to limit carbon export. (We note that in the temperate North Atlantic Ocean, for example, seasonal blooms of diatoms may utilize nearly all the silicate, then sink out leaving residual nitrate and promoting the succession of other

phytoplankton groups (ref. 38).) Given the short residence time of iron in the mixed layer, depletion of silicate will probably not occur in response to natural iron additions. It is likely that diatom growth will dominate the response to natural iron additions.

Comparison to natural systems

The two ecosystem-scale iron-enrichment experiments have produced large changes in biomass and ecosystem composition. The multiple iron additions in 1995 produced massive blooms and large drawdowns in CO_2 and nutrients. Resource utilization in these equatorial Pacific ecosystems is indeed controlled by iron availability³⁹. The resultant drawdown of carbon dioxide in the surface waters of the fertilized patch provides for a sink (or in this case, an attenuated source) for atmospheric carbon dioxide⁴⁰. These results, together with several recent studies^{3,12,14,20,37,41}, strongly support the hypothesis that iron transport to these, and other high nutrient areas, regulates carbon dioxide uptake and—at least if this occurs in the Southern Ocean—may directly influence global climate⁴⁰.

These experiments give us cause to consider how realistic these iron additions were, and if there are any natural analogues for these enrichments. Iron inputs of this magnitude are not uncommon in certain regions of today's oceans, or over wide regions of the ocean during the Last Glacial Maximum. If we assume that the input of iron during these experiments lasted about two weeks, then the flux of iron for both patch 1 and patch 3 was approximately 2 and 0.3 $\text{mmol m}^{-2} \text{yr}^{-1}$ respectively. Present-day atmospheric iron fluxes to the nitrate-limited equatorial Atlantic are of the order of 2 $\text{mmol m}^{-2} \text{yr}^{-1}$ (ref. 42). Fluxes of iron to the Pacific Ocean range from >2 $\text{mmol m}^{-2} \text{yr}^{-1}$ east of Japan to 0.02 $\text{mmol m}^{-2} \text{yr}^{-1}$ in the eastern equatorial Pacific³⁵. Our observations in coastal systems with abundant nitrate suggest that most of the particulate iron can be solubilized by iron-starved plankton (K.S.J., unpublished data). If this is the case, the fluxes of iron in our experiments are only 10–100 times higher than the ambient flux in the equatorial Pacific and lie within the range of fluxes found in the North Pacific.

Fluxes of continental dust preserved in ice cores of Greenland and Antarctica indicate a 30-fold increase in dust flux during the Last Glacial Maximum⁴³. Using isotopic tracers of export production, investigators have recently shown that there was increased ice-age carbon export to sediments of the Southern Ocean that was contemporaneous with increases of iron fluxes to these waters⁴¹. As indicated in the sediments downwind of Patagonia, the fluxes of iron to the glacial Southern Ocean increased by 5–10 times and were on the order of 5 $\text{mmol m}^{-2} \text{yr}^{-1}$. These rates of atmospheric iron delivery are similar to those used in the IronEx II experiment. Furthermore, the level of iron enrichment in patch 3 was designed to mimic the concentration of iron in the equatorial undercurrent at 140° W (ref. 23). It has been suggested that the shoaling or upwelling of this current is responsible for the increases in production along the equatorial Pacific²³. These studies indicate that these enrichment experiments were comparable with natural iron fluxes and naturally induced iron concentrations. As such, we would expect dramatic increases in equatorial Pacific primary production to occur as a result of the increased iron flux during glacial times. Even if only 20% of the iron is soluble, a 50-fold change in iron flux would be comparable to the patch 3 (+0.3 nM Fe) experiment.

Conclusions

Mesoscale iron fertilization experiments demonstrate both the feasibility and utility of manipulative experiments in the open ocean. Through these experiments there now exists a preponderance of evidence in support of the 'iron hypothesis' (that iron availability limits phytoplankton growth and biomass in the HNLC regions of the world's oceans). As this working hypothesis has been given such strong support by both experimental and palaeoceanographic observations, it is now time to regard the 'iron hypothesis' as the 'iron theory'. The natural corollary to this theory

is the suggestion that biological production in the ocean, stimulated by increased iron availability during the Last Glacial Maximum, was responsible for the observed low atmospheric levels of CO_2 (ref. 46). Such a corollary has found support in the observations of Kumar and co-workers³⁷ indicating increased export of carbon to sub-Antarctic sediments during the Last Glacial Maximum at times of higher iron flux. A present-day test of this

corollary would have its greatest significance in the Southern Ocean⁴⁰ where most of the HNLC waters are found and where the palaeoclimate coherence between iron flux and carbon export has been observed. Owing to extreme turbulence and temporal variability, a mesoscale enrichment experiment in these Southern Ocean waters poses a tantalizing, yet formidable, challenge. □

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CORRESPONDENCE should be addressed to K.H.C. (e-mail: coale@mlml.calstate.edu).

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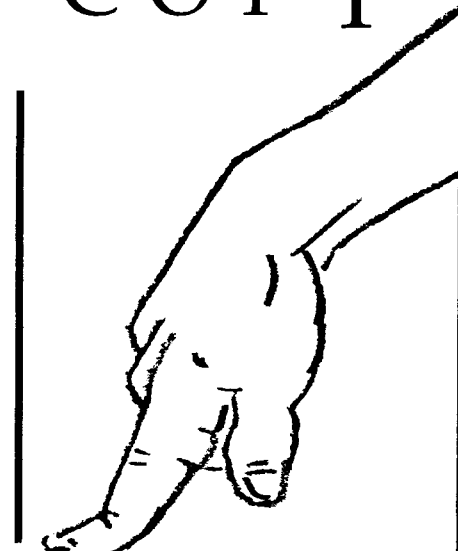
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A radio galaxy at redshift 4.41

Steve Rawlings*, Mark Lacy*, Katherine M. Blundell*,
Stephen A. Eales†, Andrew J. Bunker*
& Simon T. Garrington‡

* Astrophysics, Department of Physics, University of Oxford, Keble Road, Oxford OX1 3RH, UK

† Department of Physics and Astronomy, University of Wales, College of Cardiff, Cardiff CF2 3YB, UK

‡ Nuffield Radio Astronomy Laboratories, Jodrell Bank, Lower Withington, Macclesfield, Cheshire SK11 9DL, UK

THE most distant astronomical objects observed are quasars at redshifts of $z \approx 4.9$ (ref. 1), corresponding to a time when the Universe was less than a billion years old. This leaves little time during which the quasars and their host galaxies could form². In principle, the evolutionary state of the host galaxies can be probed by determining how many stars have formed, but this task is not straightforward because light from the quasar itself overwhelms any accompanying starlight. High-redshift radio galaxies—the likely progenitors of luminous elliptical galaxies³—provide better targets for such studies, as optical emissions from their active nuclei are observed to be faint. Here we report the discovery of a radio galaxy (6C0140 + 326) at $z = 4.41$ which shows no evidence for either a stellar continuum or an unobscured quasar nucleus. We conclude that the galaxy associated with the radio source is neither fully formed nor obviously in the process of forming stars. This implies that at least some giant elliptical galaxies are still immature at $z \approx 4.5$, and that if the intense bursts of star formation thought to produce the bulk of their stellar populations occur during the radio-bright phase, these star-forming regions are obscured by dust and gas.

There are two motivations for using radio galaxies to study stellar populations at high redshift. The first is that at low redshift they are always associated with giant elliptical galaxies⁴—the class of galaxies with the most puzzling formation history—and it is a reasonable assumption that high-redshift radio galaxies will be their progenitors. The second is that radio galaxies lack a prominent optical nucleus, a factor that makes studies of their stellar populations far easier than is the case for quasars. The discovery^{5–8} of radio galaxies at very high redshifts ($z > 3$) allowed the first studies of this type, but these have consistently been compromised by contamination problems caused by non-stellar light^{3,6–10}. The key to minimizing these problems appears to be the avoidance of galaxies of extreme radio luminosity^{8–10}, but unfortunately the two most distant radio galaxies known before this work (8C1435 + 635 at $z = 4.25$ (refs 11, 12) and 4C41.17 at $z = 3.80$ (ref. 6)) are both of extreme radio luminosity.

To probe redshifts around and above $z = 4$ while avoiding objects of extreme radio luminosity, we have undertaken redshift surveys of radio-faint samples drawn from the 151 MHz 6C survey. Following the discovery^{5,7} of two $z \geq 3$ radio galaxies in a complete sample¹³ covering 0.1 sr (with flux densities at 151 MHz S_{151} in the range $2 \leq S_{151} \leq 4$ Jy), we made the simplest assumption of a constant co-moving density $\rho \approx 10^{-9}$ Mpc⁻³ of high-redshift radio galaxies to design a follow-up survey aimed at finding them at $z > 4$. (ρ is measured in units of sources per co-moving Mpc³ per unit interval of $\log_{10} L_{151}$, with the 151 MHz luminosity L_{151} in units of $\text{W Hz}^{-1} \text{sr}^{-1}$, and with $\log_{10} L_{151} = 28 \pm 0.5$. Unless otherwise stated, we assume an Einstein–de Sitter cosmology with a Hubble constant $H_0 = 50 \text{ km s}^{-1} \text{Mpc}^{-1}$). This new survey (K.M.B. *et al.*, manuscript in preparation), again based on the 6C catalogue, covered 0.1 sr to a flux density limit $1 \leq S_{151} \leq 2$ Jy. Our spectroscopic follow-up concentrated on about 10% of the survey (~ 30 radio sources), namely those objects with radio spectral index $\alpha_{151}^{4850} \geq 1$ ($S \propto \nu^{-\alpha}$ for frequency ν between 151 and

4,850 MHz), and radio angular size $\theta < 15$ arcsec. Very distant ($z > 3$) radio galaxies invariably have these properties⁸ (the physical reasons for which are explored in refs 14 and 15 for example), so this allowed us to avoid excessive demand on optical telescope time while rejecting few high-redshift candidates. From this survey we found a radio source 6C0140 + 326 ($S_{151} = 1.00$ Jy; $\alpha_{151}^{4850} = 1.17$; $\theta = 2.6$ arcsec) at a redshift $z = 4.41$, the highest yet measured for a radio galaxy.

Observations of this galaxy (Fig. 1) were made in the following chronological sequence: (1) radio imaging with the Very Large Array (VLA); (2) blind optical spectroscopy⁷ with the William Herschel Telescope (WHT); (3) Ly α , R- and I-band imaging on the WHT; (4) near-infrared spectroscopy on the United Kingdom Infrared Telescope (UKIRT); (5) K- and H-band imaging on the UKIRT; (6) follow-up radio imaging with MERLIN and the VLA. The redshift of the galaxy is measured from a strong, wide emission line of hydrogen (Ly α) and weaker, but secure, lines of non-primordial elements (C IV 154.9 and [O II] 372.7). There is no firm detection of continuum emission from the galaxy.

We consider first the implication of a galaxy 1.6 ± 0.5 arcsec south of the eastern radio lobe of 6C0140 + 326; this galaxy is probably at $z = 0.927$ (Fig. 1) with a luminosity of $\sim L_*$ (the characteristic luminosity at the knee of the Schechter luminosity function). It is undoubtedly gravitationally amplifying 6C0140 + 326: for an L_* elliptical we expect a line-of-sight velocity dispersion $\sigma_v \approx 280 \text{ km s}^{-1}$ (consistent with the limits on the [O II] line width) and hence an Einstein ring radius $\theta_E \approx 1$ arcsec (for example, see ref. 16). The undeviated position of the eastern lobe (denoted by the angular separation θ_s from the lens) is likely to be close to the centre of the lensing galaxy, but not much less than θ_E as this would produce a counterimage (which is not seen to an image-to-counterimage ratio limit of ~ 15), obvious tangential smearing and a significant increase in flux relative to the western lobe (whereas the data shown in Fig. 1 show that the lobes have similar fluxes). This argues that $\theta_s \approx \theta_E \approx 0.8$ arcsec and leads to magnification factors of the eastern lobe, the western lobe and the Ly α cloud of $\sim 2, 1.4$, and 1.8 , respectively (denoting the average of the first two by f_{rad} , and the last by f_{opt}). Simple modelling (Fig. 1d) places the radio galaxy (and its Ly α emission) between the two radio lobes.

Accounting for this gravitational magnification, the monochromatic radio (1.5 GHz) luminosity of 6C0140 + 326 ($L_{\text{rad}} = 7.1 \times (1.7/f_{\text{rad}}) \times 10^{26} \text{ W Hz}^{-1} \text{sr}^{-1}$) is much lower than $L_{\text{rad}} = 7.6 \times 10^{27} \text{ W Hz}^{-1} \text{sr}^{-1}$ for 8C1435 + 635, the only other known $z > 4$ radio galaxy. This lower L_{rad} means that there should be less contamination of the continuum by reprocessed radiation from the active nucleus^{10,17}, so one can hope to get a more accurate picture of the stellar population of a very high-redshift radio galaxy than has previously been possible. Indeed, the faintness of the radio galaxy in the broad-band images (Fig. 1) is striking. As yet, there is no firm evidence for optical or ultraviolet continuum emission from 6C0140 + 326: the 2σ K-band limit through a 3.5-arcsec-diameter aperture is $K_{3.5} > 20.8$ mag, and the I-band detection ($I_{3.5} \approx 24$ mag) is significant only at the 2σ level. This marginal detection of rest-frame ultraviolet light (luminosity at 150 nm $L_{\text{UV}} \leq 2 \times 10^{22} \text{ W Hz}^{-1}$) is also likely to result from non-stellar processes. First, emission-line clouds emit nebular continuum and, scaling from the models of ref. 18, we find that if the Ly α /H β ratio is $\lesssim 10$ (which is true for all the high-redshift radio galaxies studied to date^{3,9}), then the predicted nebular continuum luminosity approaches the value of L_{UV} . Second, although not extreme, the L_{rad} of 6C0140 + 326 is still comparable to those of the $z \geq 1$ 3C galaxies in which a flat-spectrum (optical spectral index $\alpha_{\text{opt}} \approx 0$) component often dominates the ultraviolet light¹⁰; this component appears to be induced by the radio activity¹⁰, and measurements of its polarization⁸ prove that it is non-stellar in at least some cases. If this component scales with L_{rad} (refs 10, 17), then its predicted ultraviolet luminosity ($\sim 10^{22} \text{ W Hz}^{-1}$ at 150 nm,

excluding any correction for lensing) again approaches the value of L_{UV} . The flat spectra of both non-stellar components means that their contribution is less important at longer wavelengths.

In Fig. 2 we consider limits on the spectral energy distribution of the starlight from 6C0140 + 326. If $\Omega_M = 1$ (Fig. 2a), then 6C0140 + 326 can only be a mature elliptical galaxy at its observed

redshift if it has a luminosity close to or below L_* : synthetic photometry of an L_* elliptical evolved to $z = 4.41$ (Fig. 2) gives $K \approx 21.2$ mag. This is a significantly lower stellar luminosity than one might expect from studies of radio galaxies at $z \approx 0$ and $z \approx 1$ (and lower also than those inferred for 8C1435 + 635 and 4C41.17; see Fig. 2). A similar argument has previously been

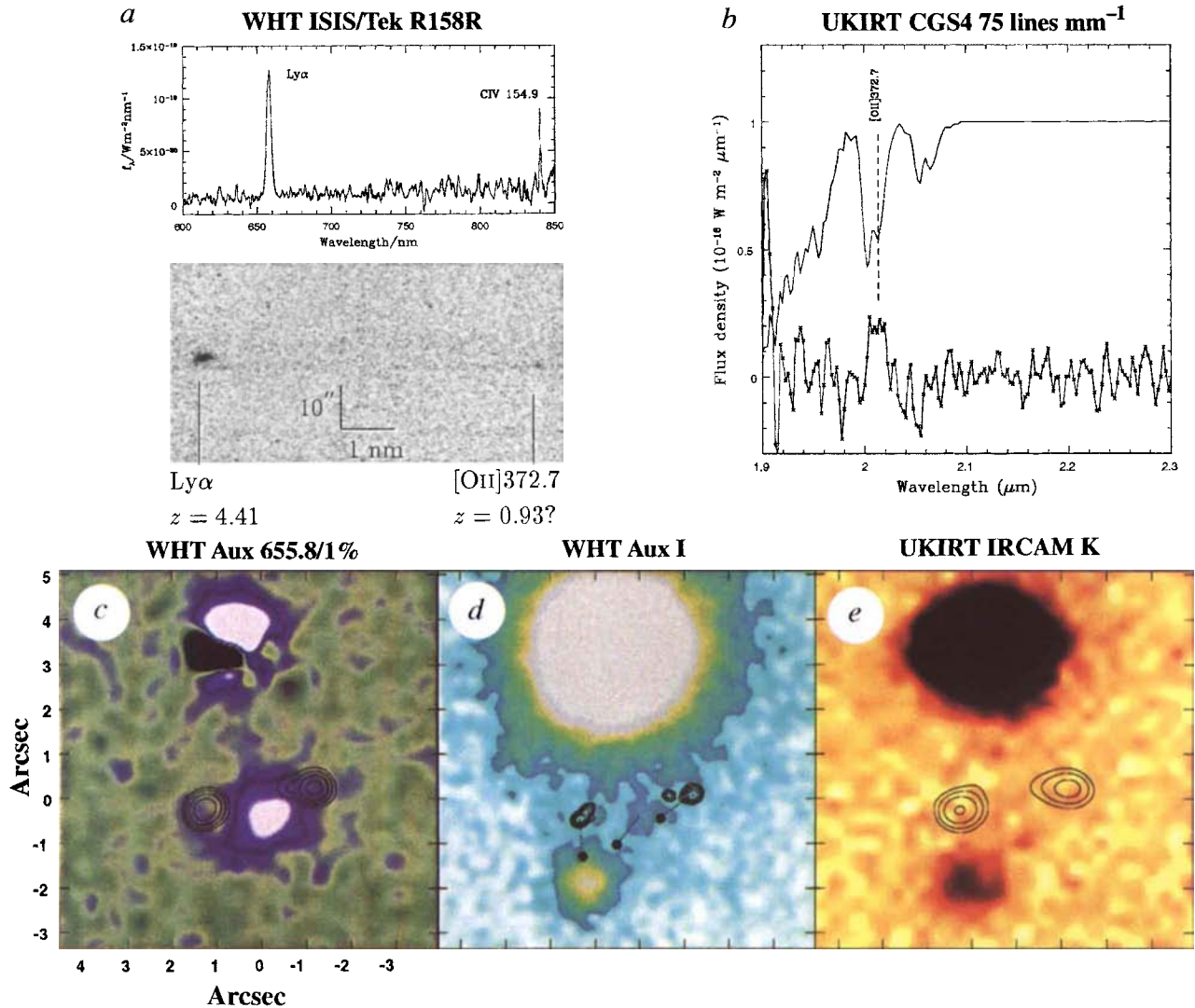


FIG. 1 Spectra and images of 6C0140 + 326 taken at or near RA(1950) 01 h 40 min 51.5 s, Dec.(1950), +32° 38' 45.7" (the origin of the images registered adopting a position, accurate to ± 0.5 arcsec, of 01 h 40 min 51.56 s, +32° 38' 49.1" for the bright star north of the radio source). a, Optical spectrum. Top, data extracted at the target position from an average of two 1,800-s exposures taken at PA 192° and PA 134°, smoothed with a 5-pixel box filter. The Ly α line peaks at 657.9 ± 0.2 nm, has a flux within $\pm 20\%$ of 4.5×10^{-19} W m⁻² through a 2×2 arcsec² aperture, and a deconvolved full width at half-maximum (FWHM) of $\sim 1,500$ km s⁻¹. The C IV 154.9 line is ~ 10 times weaker but securely detected at ~ 840.2 nm in separate frames. Bottom, some of the two-dimensional spectrum at PA 134°, showing the Ly α line from the radio galaxy, and continuum and a single emission line at 718.3 ± 0.3 nm (FWHM < 500 km s⁻¹ and flux $\approx 8 \times 10^{-20}$ W m⁻²) from the foreground galaxy. The line is most likely to be [O II] 372.7 at $z = 0.927$, so its magnitudes ($K_{3.5} = 18.5 \pm 0.15$ mag; $H_{3.5} = 19.8 \pm 0.2$ mag; $I_{3.5} = 21.4 \pm 0.2$ mag; $R_{3.5} = 22.5 \pm 0.15$ mag) and structure imply it is an elliptical with luminosity $\approx L_*$ (we take $M_* = \pm 21.2$ in the B_J photometric band from ref. 32). b, Infrared spectrum centred on the radio galaxy (lower curve); a secure line at 2.015 ± 0.005 μ m is redshifted [O II] and has flux within $\pm 40\%$ of 3×10^{-19} W m⁻² through a 2.4×2.4 arcsec² aperture; the line width was unreliable because of the difficulty correcting for atmospheric transmission (upper curve). c, A grayscale of the Ly α image with the VLA 5

GHz radio contours overlaid on it. The flux in a 3-arcsec-diameter aperture enclosing the Ly α emission is 8.6×10^{-19} W m⁻², consistent with it dominating the R-band magnitude of 23.8; an R-band image has been scaled and subtracted from the narrow-band image. d, I-band image overlaid with a 1.66 GHz MERLIN map. The lines connect the positions of the radio components and the galaxy with those predicted in the absence of gravitational lensing; we adopted a singular isothermal sphere model¹⁶ for the galaxy (as implemented by the GLENS task in the AIPS software package) with $\theta_E = 0.8$ arcsec, and varied the undeviated position to the point where the predicted counterimage fell below our detection limit. e, K-band image overlaid with an 8-GHz VLA map; the flux and position of a highly marginal K-band feature ~ 1 arcsec south of the radio midpoint is consistent with it being entirely the [O II] 372.7 line in Fig. 1b. The radio contours are all spaced logarithmically by factors of two, with the VLA 5-GHz and MERLIN maps starting at 0.5 mJy per beam, and the VLA 8-GHz map at 0.25 mJy per beam; the synthesized beam has a diameter of 0.46 arcsec on the VLA maps, and 0.16 arcsec on the MERLIN map. From our VLA maps, we measure total radio flux densities (in mJy) of 76.83 ± 0.58 , 17.13 ± 0.52 , 8.12 ± 0.27 and 3.0 ± 0.67 at 1.5, 4.8, 8.4 and 15.0 GHz respectively; the corresponding values for the east and west lobes are $(35.68 \pm 0.33, 9.19 \pm 0.31, 4.41 \pm 0.16, 0.9 \pm 0.2)$ and $(39.32 \pm 0.33, 7.49 \pm 0.31, 3.17 \pm 0.16, 1.7 \pm 0.16)$.

used to support a young galaxy interpretation for the $z = 3.4$ radio galaxy B20902 + 34 (refs 19, 20; Fig. 2), and our study of 6C0140 + 326 provides further evidence that either luminous radio sources can form in less massive galaxies at early cosmic epochs, or that the stars in the central regions of some high-redshift radio galaxies are not yet in place, not yet formed, or hidden by dust. The apparent lack of centrally concentrated stars in 6C0140 + 426 and B20902 + 34, together with the evidence for a diffuse stellar halo in B20902 + 34 (refs 19, 20) may be telling us something about the formation of elliptical galaxies (for example, that the central stars are the last to form, as suggested by the metallicity gradients observed in nearby ellipticals²¹), or may instead reflect the distribution of dust. In either case, the implication is that if these are giant ellipticals then they are immature, a result that is certainly in line with the short time available at $z > 4.4$ in the Einstein–de Sitter cosmology.

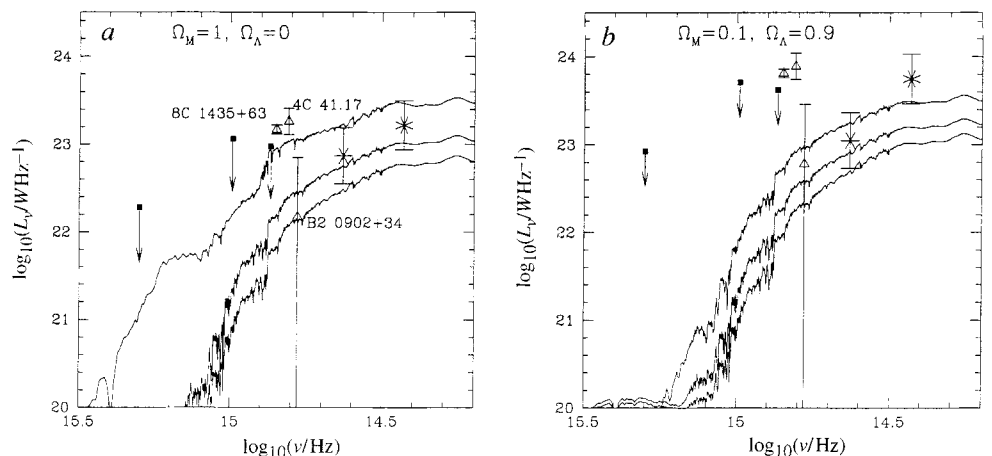
The apparent lack of centrally concentrated stars in 6C0140 + 426 follows from two features of our assumed cosmological model: (1) the redshift dependence of luminosity; and (2) the large decrease in stellar luminosity expected over the range $0 < z < 4.4$ due to passive evolution—the dimming and reddening of a post-starburst population as the main-sequence turn-off evolves (curves in Fig. 2a). It is possible, therefore, to retain the notion that 6C0140 + 426 is a mature giant elliptical if one is prepared to consider other models, for example: a reduction in the influence of passive evolution over $0 < z < 4.4$ by taking $H_0 < 50 \text{ km s}^{-1} \text{ Mpc}^{-1}$; or a low-density alternative to the Einstein–de Sitter model (Fig. 2b).

We have also considered other possible indicators of the status of the stellar population of 6C0140 + 326. It is almost certainly incorrect to argue for a huge star-formation rate (SFR) ($\sim 1,000 M_\odot \text{ yr}^{-1}$) on the basis of the [O II] luminosity $L_{\text{[OII]}}$ (for example, by naive implementation of the formula of ref. 22), because the extreme line equivalent widths (a rest-frame value $> 40 \text{ nm}$ in the case of Ly α) and velocity widths ($\sim 1,500 \text{ km s}^{-1}$) argue strongly against star formation as the source of photoioniz-

ing radiation: 6C0140 + 326 lies on the $L_{\text{[OII]}} - L_{\text{rad}}$ correlation (for example, see refs 8, 17), so $L_{\text{[OII]}}$ is probably derived from the action of the radio source and/or a hidden quasar nucleus. Moreover, recalling that the Ly α is spatially resolved, the large velocity width of the line is very unlikely to be due to gravitationally induced motions and probably reflects the speeds of shocks driven into the line-emitting gas by the radio source⁶. We can nevertheless use L_{UV} to set a limit on the SFR of $< 40 \times (1.8/f_{\text{opt}}) M_\odot \text{ yr}^{-1}$ (ref. 23). If one accepts this limit (which is robust if there is no obscuration of star-forming regions by dust and gas) and also the notion that radio galaxies are associated only with massive ellipticals, then a somewhat contrived star-formation history is suggested. As the product of the age of the Universe at $z = 4.41$ ($\sim 10^9 \text{ yr}$) and the SFR gives a mass in stars significantly less than seen in an L_* elliptical, one can first deduce that we are not probing the redshift at which the SFR peaks. If this peak occurs at $z > 4.4$, it must be of short duration ($< 10^9 \text{ yr}$, given the cosmic time available) and intense (SFR $\approx 1,000 M_\odot \text{ yr}^{-1}$ to form the stars of a giant elliptical), but this rate must drop by the time of the radio-loud phase, with subsequent starbursts and/or dynamic evolution³ to explain the apparently low luminosity in centrally concentrated stars. If the peak SFR occurs at $z < 4.4$, then the starburst is constrained to be just as short and intense by the evolved stellar populations of radio-selected ellipticals at $z \approx 1$ (refs 3, 24), but again the SFR must be low during the radio activity, and the nuclei of galaxies must be capable of producing powerful radio jets before they form most of their stars (contrary to expectations: see ref. 2 for example).

We conclude by exploring other possible explanations of the observed properties of 6C0140 + 426. In this regard it is interesting that optical estimates of the SFR in all high redshift galaxies found to date^{23,25,26} are fairly moderate ($\sim 10 M_\odot \text{ yr}^{-1}$), and in some cases²⁵ insufficient to form an L_* galaxy by the redshift at which the galaxy is observed, just as we have argued for 6C0140 + 426. If these galaxies are precursors of disk galaxies, then a moderate

FIG. 2 The rest-frame spectral energy distribution (SED) of 6C0140 + 326 in comparison with lower-redshift elliptical galaxies extrapolated to high redshift using a stellar spectral synthesis model (kindly supplied by G. Bruzual³³). In a, we assume an Einstein–de Sitter cosmology ($\Omega_M = 1$, and a dimensionless cosmological constant, $\Omega_\Lambda = 0$); in b, we assume $\Omega_M = 0.1$ and $\Omega_\Lambda = 0.9$ (which is also a reasonable representation of the behaviour in an $\Omega_\Lambda = 0$ low-density model); in both cases we take $H_0 = 50 \text{ km s}^{-1} \text{ Mpc}^{-1}$. The square data points are plotted as 2σ limits: for a circular aperture of diameter θ arcsec, these are $I > (25.4 - 2.5 \log_{10} \theta) \text{ mag}$, $H > (22.5 - 2.5 \log_{10} \theta) \text{ mag}$ and $K > (22.2 - 2.5 \log_{10} \theta) \text{ mag}$; we plot the marginal detection in I-band as a limit on stellar luminosity for reasons discussed in the text. Because of imperfect subtraction of the lensing galaxy and light spillage from the star at large diameters, we take $\theta = 3.5$, which corresponds to ~ 20 and $\sim 45 \text{ kpc}$ in cases a and b respectively. We cannot correct precisely for the effects of gravitational lensing, but note that for an aperture of fixed angular size, surface brightness conservation will lead to more flux (by a factor between 1 and $f_{\text{opt}} = 1.8$) within a central metric diameter (smaller by $\sim f_{\text{opt}}^{0.5}$) if we make the reasonable assumption that surface brightness falls monotonically with radius. In both a and b, the SEDs expected for an L_* elliptical at redshifts 4.41, 1 and 0 are plotted as the upper, middle and lower curves respectively. The spectral synthesis model³³ assumes that all the stars were formed *in situ* (with a Salpeter initial mass function with cutoffs at $0.1 M_\odot$ and $125 M_\odot$) in a short (10^8 yr) burst at extreme redshift ($z = 100$); in the context of models that neglect dynamical evolution, this makes a model



post-starburst galaxy as dim as possible by $z = 4.41$ (ref. 3). The models are normalized to match an L_* elliptical at low redshift with the assumption that 20- and 45-kpc metric apertures include 50 and 75% of the light, respectively. The six-pointed asterisk (and accompanying error bar) shows the range in stellar luminosity that encompasses $\sim 90\%$ of low-redshift 3C radio galaxies⁴ (with an aperture correction); the eight-pointed asterisk (and error bar) shows the corresponding range for 3C and 6C radio galaxies at $z \approx 1$ (calculated from aperture-corrected K magnitudes from ref. 24). Also plotted (triangles in a) are the optical luminosities of three other high-redshift radio galaxies discussed in the text; data, including corrections for emission-line contamination, are taken from refs 6 and 9 for 4C41.17 ($z = 3.80$), from ref. 19 for B20902 + 34 ($z = 3.40$), and from refs 11 and 12 for 8C1435 + 635 ($z = 4.25$).

SFR at high redshift is consistent with the relatively low mass and ongoing low-redshift star formation of this class of galaxy, but this is not the case if any are precursor giant ellipticals, as we contend is likely for objects like 6C0140 + 426. One possibility that allows a more intense starburst than optical measurements imply and also allows it to be synchronized with the radio activity, is suggested by far-infrared and millimetre detections of large dust and gas masses in high-redshift radio galaxies^{27,28} and quasars^{29,30}—some of the stars, and much of the star formation, may be hidden by the dust and gas present in young galaxies. A second possibility is that there is more time available at high-redshift owing either to a very low value of H_0 ($< 50 \text{ km s}^{-1} \text{ Mpc}^{-1}$) or a cosmology that differs from Einstein–de Sitter; this has been given some credence by the recent discovery³¹ of a radio galaxy at $z = 1.55$ whose stellar population appears to have an age worryingly close to that of an $H_0 = 50 \text{ km s}^{-1} \text{ Mpc}^{-1}$ Einstein–de Sitter Universe at this redshift. □

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CORRESPONDENCE should be addressed to S.R. (e-mail: s.rawlings1@physics.oxford.ac.uk).

Peptide oligomers for holographic data storage

Rolf H. Berg, Søren Hvilsted & P. S. Ramanujam

Risø National Laboratory, DK-4000 Roskilde, Denmark

SEVERAL classes of organic materials (such as photoanisotropic liquid-crystalline polymers^{1–4} and photorefractive polymers^{5–7}) are being investigated for the development of media for optical data storage. Here we describe a new family of organic materials—peptide oligomers containing azobenzene chromophores—which appear particularly promising for erasable holographic data storage applications. The rationale for our approach is to use the structural properties of peptide-like molecules to impose orientational order on the chromophores, and thereby optimize the optical properties of the resulting materials. Here we show that holographic gratings with large first-order diffraction efficiencies (up to 80%) can be written and erased optically in oligomer films only a few micrometres thick. The holograms also exhibit good thermal stability, and are not erased after heating to 180 °C for one month. Straightforward extension of this peptide-based strategy to other molecular structures should allow the rational design of a wide range of organic materials with potentially useful optical properties.

Optical storage in azobenzene-containing polymers is based on laser-induced photoisomerization of the chromophore. When irradiated in a certain wavelength range the azobenzene undergoes random reorientations through a number of *trans*–*cis*–*trans* isomerization cycles. A stationary orientation is obtained when the optical transition moment, which lies approximately parallel to the long axis of the *trans*-azobenzene, is oriented in the plane perpendicular to the polarization direction of the laser beam^{3,8}. This anisotropic alignment of azobenzenes, which leads to a local modulation of the refractive index, forms the basis for the storage process. We have tried to increase the molecular anisotropy by preorganizing the chromophores so as to reduce the number of stationary orientations possible.

We designed oligomers, or diamino acid-*N*²-substituted oligopeptides (DNOs), in which azobenzene side chains were linked to

a peptide-like backbone. This backbone was expected to impose helical stacking of the azobenzenes in a manner similar to that found for the bases in DNA or PNA^{9–12} (Figs 1 and 2). The particular DNO backbone shown in Fig. 1 is similar to that of PNA, and consists of all-*L* ornithine units oligomerized through the δ -amino groups and with the side chains attached to the α -amino groups by carbonyl linkers. Glycine was chosen as the *N*-terminal backbone unit, and a dipolar cyanoazobenzene derivative^{1–3,13–16} was chosen as the chromophore. A conceptual model that illustrates the rationale behind the DNO design is shown in Fig. 2. A single azobenzene, in which the transition moment of the chromophore lies along the *y*-axis, can be rotated in the entire *x*–*y* plane and in any position around the *y*-axis, while retaining its transition moment orientated perpendicular to the polarization, $\hat{E}$, of the laser beam. In the case of the ornithine-based DNO dimer, it is assumed that the two neighbouring azobenzenes are stacked, so any rotation around the *y'* or the *y''* axis will rotate both of them, resulting in a rotation of the entire molecule. Assuming that the two azobenzenes are stacked in a helical manner, any such rotation, other than 180°, would lead to a situation in which the transition moment of the neighbouring azobenzene is no longer oriented perpendicular to $\hat{E}$. Accordingly, the molecule would still

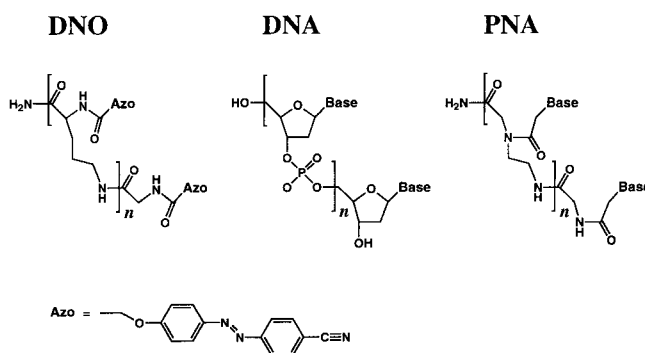


FIG. 1 Chemical structures of the DNO oligomer, DNA and PNA. The length of the *trans*-azobenzene chromophore shown is on the same order as that of a Watson–Crick base pair in double-helical DNA ($\sim 10 \text{ \AA}$).

be able to undergo photoisomerization cycles. In other words, with the DNO dimer or longer oligomers, a stationary orientation will not be obtained until the planes of the azobenzenes are oriented perpendicular, or roughly perpendicular, to $\vec{E}$. As a result, there are considerably fewer stationary orientations possible than in cases where the azobenzenes are either not stacked or are stacked parallel to one another.

Merrifield synthesis^{17,18} of DNO oligomers proceeded without difficulties from the readily available 'submonomers' (see Methods). Films of good optical quality with a thickness of approximately 5 μm were obtained from hexafluoroisopropanolic solutions. Polarization microscopy indicated that the films were isotropic. To test its use for optical storage applications, holographic gratings were recorded in a film of DNO dimer **1** (Fig. 3a), and a polarization holographic technique was used to record the gratings. It has been shown that by using two orthogonally circularly polarized beams to write a grating and a circularly polarized beam for the read-out, 100% efficiency can theoretically be obtained in one first-order diffracted beam, even for thin gratings¹⁹. In the present case, the light of wavelength 488 nm from an argon ion laser at a total intensity of 2 W cm⁻² was used as the writing beam, and a 4.2-mW circularly polarized beam at 633 nm from a He-Ne laser was used for read-out. A first-order diffraction efficiency of 76% was reached in about 300 s (Fig. 3a). This corresponds to a diffraction efficiency near the maximum achievable, because absorption losses, reflection losses, and diffraction in other orders account for approximately 24%; surface grating formation may have contributed to the diffraction in other orders. After 300 s, one of the circularly polarized beams is switched on; the diffracted power decreased rapidly and reached a level of 0.05 mW after 300 s, resulting in erasure of about 98% of the grating. The holograms can be rewritten and erased several times without fatigue. A much lower diffraction efficiency (15%) is obtained with the monomer **M**, which contains only a single azobenzene side chain (Fig. 3a).

The effect of oligomer size is examined in Fig. 3b. The writing time (that is, the rate of formation of holographic gratings) accelerates with increasing size of the DNO oligomer, implying the existence of cooperative dynamic effects between neighbouring azobenzenes. Preliminary experiments indicate that about ten residues may be an optimal size. To examine the effects of structurally modifying the side chains of dimer **1**, analogues

were prepared in which the linker from the backbone to the azobenzene ligand is extended by two, three and four methylene groups, respectively. The diffraction efficiency is lowered gradually when the number of methylene groups is increased (Fig. 3c). For the most extended analogue, **7**, a maximum value of only 3% was obtained. These results indicate that increasing the flexibility of the linker between the backbone and the azobenzene ligand reduces the ability of the backbone to impose stacking of the azobenzenes. The backbone was modified to examine further the variability of the DNO structure. Substituting the achiral glycine unit of dimer **1** with an L-alanine unit (**8**; Fig. 3d) was found to shorten substantially the writing time. Taken together with the inability of a D-alanine-substituted analogue (**9**; Fig. 3d) to reach more than 25% diffraction efficiency, these results indicate that proper stacking is favoured when the azobenzenes are linked to backbone units with the same directionality. The effect of varying the number of bonds in the backbone is examined in Fig. 3e. The writing time is accelerated markedly for dimers based on the shorter diaminopropionic acid (four bonds) and diaminobutyric acid (five bonds) compared with dimers based on ornithine (six bonds) and lysine (seven bonds). It seems that the molecular geometry of the shorter backbones also allows neighbouring azobenzenes to stack in a proper manner, and with stronger stacking interactions, owing to their closer proximity. The effect of oligomer size is examined in Fig. 3f for diaminobutyric acid-based oligomers. Again, it is seen that the writing time accelerates with increasing oligomer size. With DNO decamer **15**, a first-order diffraction efficiency of 78% is reached in about 10 s. Work in progress shows that short DNO oligomers can be linked together in such a way as to achieve writing times considerably shorter than those of this study.

At present, only limited information about the structure of DNO films has been obtained. As has been seen in other azobenzene-containing systems^{13,16}, Fourier transform infrared (FTIR) polarization spectra of laser-irradiated films showed a preferred orientation of the chromophore transition moment that is perpendicular with respect to the laser polarization. The structure of DNO oligomers in the film state should bear some resemblance to the structure of oligomers dissolved in hexafluoroisopropanol. The circular dichroism spectrum of an ornithine-based decamer, measured in hexafluoroisopropanol, in the azobenzene absorption region, is distinct from that of the monomer, and the pronounced circular dichroism observed with the decamer suggests the existence of close interactions between neighbouring chromophores oriented in a helical manner relative to each other²⁰.

Holograms written in DNO oligomers are stable at room temperature, and lifetimes close to one year have been achieved. Surprisingly, the holograms are also exceptionally stable to heat, not being erased after exposure to 180 °C for one month, and the films can be reused. This extraordinary stability may be reflected in the thermal behaviour of the materials, as no thermal transitions were detected, by differential scanning calorimetry, in the temperature range from 25 °C to the decomposition temperature. The materials examined in this study decomposed between 210 °C and 230 °C. Nonetheless, holograms written before heating are still weakly present after exposure to 250 °C for one month.

Work currently underway suggests that DNO oligomers might also prove valuable for volume holography²¹. Thick films of DNO oligomers, immobilized within the bulk of a 65- μm -thick polystyrene-grafted polyethylene film²², have been fabricated, resulting in diffraction efficiencies of 20%. A more appropriate choice of polymer composition should allow the fabrication of even thicker films and higher levels of efficiency.

In previous photoanisotropic organic materials, diffraction efficiencies near the maximum achievable have been obtained, to the best of our knowledge, only in two metastable systems. In one case a large (~5 kV) voltage was applied across a ~100- μm -thick film of a photorefractive polymer composite⁶, and in the second case a ~30- μm -thick film of a thermally quenched liquid-crystalline polymer was used²³.

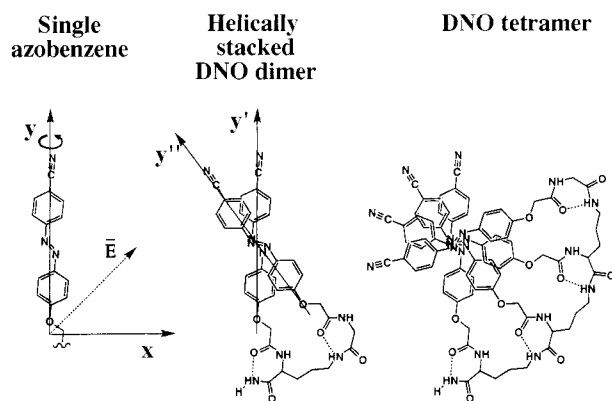


FIG. 2 Helically stacked azobenzenes, in contrast with single ones (or azobenzenes stacked in a parallel manner), cannot be rotated freely around their transition moment axis (for example, y'), while still retaining the DNO molecule in a stationary orientation. The DNO dimer and tetramer models are viewed from the top and are drawn with the normal DNA (B-DNA) helical twist of 36° between neighbouring azobenzenes. Examination of a DNO model suggests that specific hydrogen bonds, such as those shown here, could play a structural role in predefining the stacking of the azobenzene side chains.

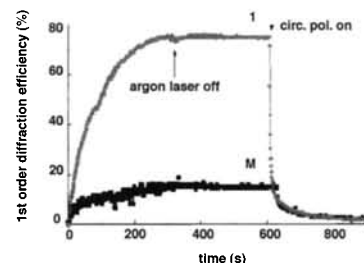
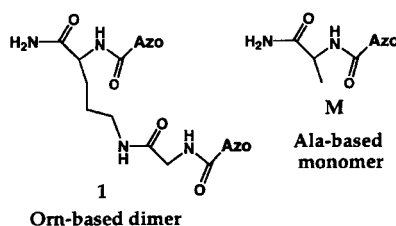
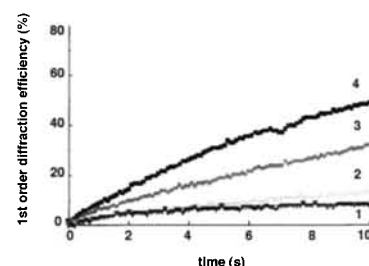
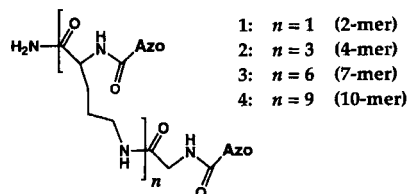
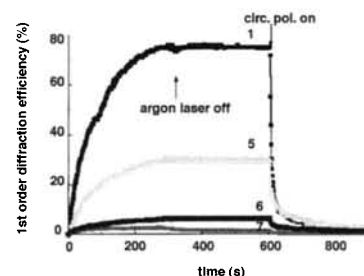
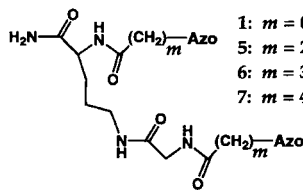
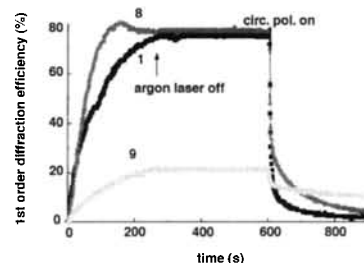
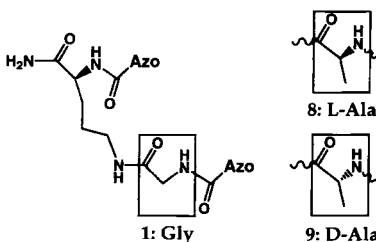
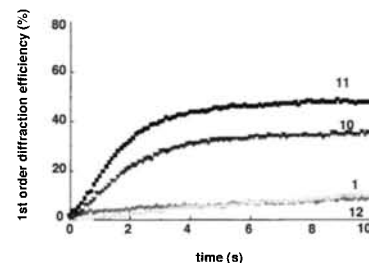
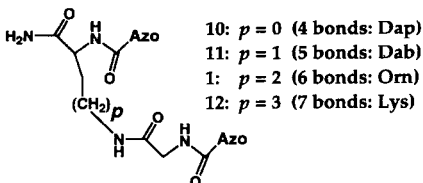
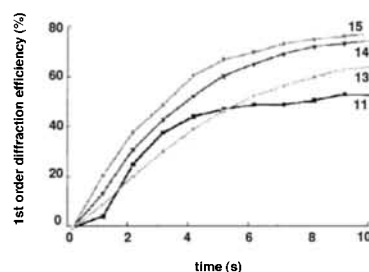
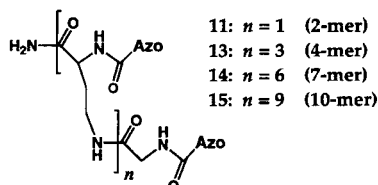
a Dimer versus monomer**b** Effect of oligomer size**c** Effect of side-chain flexibility**d** Effect of stereochemistry**e** Effect of number of bonds in the backbone**f** Effect of oligomer size (Dab)

FIG. 3 First-order diffraction efficiencies as a function of time, measured during the grating formation in $\sim 5\text{-}\mu\text{m}$ -thick films of different DNO oligomers. *a*, Ornithine-based dimer **1** versus alanine-based monomer **M**. For **1**, the diffracted power in the first order increases in about 300 s to 3.2 mW, representing a first-order diffraction efficiency of 76%. Diffracted power in the -1 order accounted for 1%. The diffraction efficiency remains stable after the argon laser is switched off. *b*, Dimer **1** versus longer analogues. *c*, Ornithine-based dimers with increasing number of methylene groups between the backbone and the azobenzene chromophore. *d*, Ornithine-based dimers where one backbone unit (L-ornithine) is combined with a second backbone unit, which is either achiral (glycine, **1**) or has the same (L-alanine, **8**) or the opposite chirality (D-alanine, **9**). *e*, Dimers with increasing number of methylene groups in the backbone based on diaminopropionic acid (**10**), diaminobutyric acid (**11**), ornithine (**1**) and lysine (**12**), respectively. *f*, Diaminobutyric acid-based dimer versus longer analogues.

Our studies of DNO oligomers have demonstrated a general and versatile approach to the design of new, and potentially very useful, materials for holographic storage. □

Methods

DNO synthesis. Merrifield synthesis of the DNO oligomers involved three levels of protection, using *tert*-butoxycarbonyl (Boc)²⁴ as a weak acid-labile backbone amino-protecting group, 9-fluorenylmethoxycarbonyl (Fmoc)²⁵ as a weak base-labile α -amino-protecting group, and a benzhydrylamine derivative as a strong acid-labile anchoring linkage to the solid support²⁶. The solid support of choice was *p*-methylbenzhydrylamine-copoly(styrene-1%-divinylbenzene) resin²⁷. Each cycle of the solid-phase assembly consists of two individual coupling steps: the first step introduces the backbone unit, and the second incorporates the side-chain unit. Oligomers containing two or more residues were synthesized without difficulties. The direction of the solid-phase synthesis was from the C terminus to the N terminus. Unless otherwise stated, glycine was used as the N-terminal backbone unit. As an example of such a synthesis, the DNO decamer **4** was synthesized using the commercially available backbone monomer *N*²-Fmoc-L-Orn(*N*³-Boc)-OH (where Orn is ornithine) and the azobenzene side-chain monomer NC-C₆H₄-N=N-C₆H₄-O-CH₂-COOH, prepared by basic hydrolysis of the corresponding ethyl ester, which in turn is prepared in a Williamson ether coupling of 4-(4-cyanophenylazo)phenol and ethyl bromoacetate. Boc-Gly-OH was used to incorporate Gly. To obtain a C-terminal amide, the oligomer was assembled on the benzhydrylamine-type resin, initially loaded with 0.45 mmol of *N*²-Fmoc-L-Orn(*N*³-Boc) per gram resin. Activation of 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU), in combination with *in situ* neutralization, allowed efficient incorporation of the backbone residues when the couplings were performed in 50% (v/v) pyridine in *N,N*-dimethylformamide with a monomer concentration of 0.10 M. The side-chain units were incorporated by *in situ* diisopropylcarbodiimide activation of the tetrabutylammonium salt of the azobenzene side-chain monomer in 50% methylene chloride in *N,N*-dimethylformamide with a monomer concentration of 0.10 M in the presence of the weak acid 3,4-dihydro-3-hydroxy-4-oxo-1,2,3-benzotriazine (DHBT-OH) (5 equivalents). The alternating deprotections of *N*²-Fmoc and *N*³-Boc groups were accomplished with 20% piperidine in *N,N*-dimethylformamide and 50% trifluoroacetic acid in methylene chloride, respectively. The progress of the synthesis was monitored throughout by the quantitative²⁸ Kaiser ninhydrin reaction²⁹, which indicated that the double coupling (2 × 30 min) of each backbone residue and the single coupling (1 × 30 min) of each side-chain residue proceeded with an overall coupling yield of >95%. Cleavage of the free oligomer from the resin was accomplished with neat anhydrous HF at 0 °C for 1 h. The DNO oligomer was extracted from the resin with 50% trifluoroacetic acid in methylene chloride, and was obtained as a reddish powder after evaporation of the solvents and drying in vacuum at 90 °C overnight.

DNO films. DNO oligomers are insoluble in most solvents, but are readily dissolved in trifluoroacetic acid or hexafluoroisopropanol. For preparation of films of good optical quality, the DNO oligomer in question was dissolved in methylene chloride:trifluoroacetic acid:hexafluoroisopropanol (5:20:75 by volume), filtered through a fine-grade glass filter, cast onto a glass substrate, and immediately dried in vacuum for 1 h. Finally, the cast film was dried at 90 °C overnight.

Holography. For holographic experiments, the beam from the argon ion laser was split by means of a polarization beam splitter, converted to orthogonally circularly polarized beams using appropriate quarter-wave plates. The beams were allowed to overlap on the film, giving a spatial resolution of about 510 lines per mm, corresponding to a grating spacing of about 1.96 μ m; a resolution of about 3,000 lines per mm, corresponding to a grating spacing of about 0.33 μ m, has been achieved. At the writing wavelength (488 nm) and the reading wavelength (633 nm), the linear absorption coefficient of the samples was about 3,000 and 200 cm⁻¹, respectively.

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CORRESPONDENCE should be addressed to R.H.B. (e-mail: rolf.berg@risoe.dk).

Confirmation of iron limitation of phytoplankton photosynthesis in the equatorial Pacific Ocean

Michael J. Behrenfeld*, Anthony J. Bale†, Zbigniew S. Kolber*, James Aiken† & Paul G. Falkowski*

* Oceanographic and Atmospheric Sciences Division, Brookhaven National Laboratory, Upton, New York 11973-5000, USA

† NERC Plymouth Marine Laboratory, Prospect Place, West Hoe, Plymouth PL1 3DH, UK

THE eastern equatorial Pacific Ocean is one of only three open-ocean regions where low phytoplankton chlorophyll biomass persists despite perennially high nitrate and phosphate nutrient concentrations¹. In 1993, an area within this region was artificially enriched with a single dose of soluble iron to test whether phytoplankton are physiologically prevented from utilizing the available nutrients by the low natural iron concentrations^{2,3}. Although photosynthesis was stimulated⁴, the observed lack of a bloom or a significant decrease in nutrient concentrations could not be attributed unequivocally to zooplankton grazing^{5–7}, further iron limitation or secondary nutrient limitation^{2,4}. In 1995, a second iron-enrichment experiment (IronEx II) was conducted in which the same total dosage of iron was added, but over eight days⁸. A massive phytoplankton bloom developed, significantly reducing surface-water nutrient and CO₂ concentrations^{8–10}. Here we report *in situ* measurements of fluorescence during IronEx II, which show that the iron enrichment triggered biophysical alterations of the phytoplankton's photosynthetic apparatus, resulting in increased photosynthetic capacities throughout the experiment and, hence, the observed bloom. These results unequivocally establish physiological limitation of phytoplankton by iron as the cause of the high-nitrate, low-chlorophyll phenomenon in this ocean region.

A 64 km² region of the eastern equatorial Pacific Ocean was enriched with ferrous sulphate to a final concentration of 2 nM iron on 29 May, followed by two 1 nM iron enrichments on 1 and 5 June 1995⁸. The effects of iron enrichment were detected in real time by following changes in variable fluorescence parameters^{11,12}. From these measurements, we deduced changes in photochemical quantum efficiencies (F_v/F_m) and functional absorption

cross-sections (σ_{PSII}) of photosystem II^{11,12}. Before iron enrichment, the study site was dominated by picoplankton, primarily *Prochlorococcus* sp. and *Synechococcus* sp.¹³, with uniformly low F_v/F_m within the upper 40 m (Fig. 1a). During the first 25 h following iron enrichment, F_v/F_m increased exponentially from 0.25 to 0.50 (Fig. 2). The high temporal resolution of these measurements revealed the initial *in situ* response of the natural phytoplankton population to alleviation of iron limitation. This immediate increase in F_v/F_m occurred before any changes in species composition¹³ and demonstrated that the extant phytoplankton assemblage was physiologically stimulated by micro-nutrient enrichment and that this response requires an intrinsic alteration in the photosynthetic apparatus within the phytoplankton community. Such a response would not be observed if

production of the autotrophic population (that is, the rate of carbon fixation by photosynthesis) was limited by extrinsic factors such as grazing.

A conspicuous lens of physiologically stimulated cells developed within 32 h of the initial iron enrichment, spanning 10 km horizontally and penetrating to >25 m depth (Fig. 1b). As observed during the earlier *in situ* iron-fertilization experiment (IronEx I)⁴, F_v/F_m peaked on the second day and then began to decline (Fig. 3). In IronEx II, however, elevated values of F_v/F_m were renewed by a second iron enrichment, suggesting that the reductions in F_v/F_m during IronEx I were due to the loss of available iron rather than to limitation by a secondary micro-nutrient or grazing. With the aid of a third enrichment, an elevated F_v/F_m was maintained for 8 days (Figs 2 and 3), during which a mixing event deepened the lens of physiologically stimulated cells to >40 m (Fig. 1c). Although biomass remained high for 13 days, a gradual decrease in F_v/F_m ensued after day 8 until pre-enrichment values were once again measured (Fig. 3).

Increases in F_v/F_m were accompanied by a doubling of σ_{PSII} during the first 25 h (Fig. 2, inset). The response of σ_{PSII} to iron enrichment is dependent upon the initial growth irradiance and the degree of iron limitation^{4,14-16}. The rapid increase in σ_{PSII} during IronEx II is a characteristic response to iron enrichment in phytoplankton with functionally isolated photosynthetic units and limited capacity for photon energy transfer¹⁴⁻¹⁶, and suggests

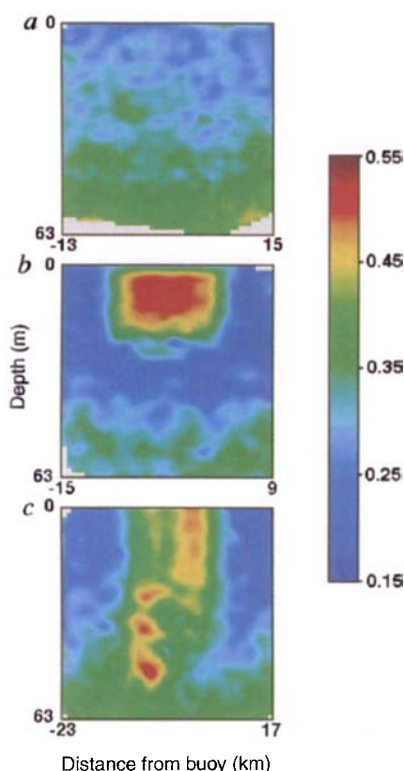


FIG. 1 Changes in photochemical quantum efficiencies (F_v/F_m) in the upper 65 m of the water column resulting from the addition of nanomolar concentrations of iron. The inert tracer, SF_6 , was added with ferrous sulphate to permit independent detection of the enriched region. In addition to SF_6 , a langrangian drifting buoy was deployed for orientation within the enrichment area. a, Uniformly low F_v/F_m in the upper 40 m existed in the study region before iron addition. b, 32 h after iron enrichment, a lens of enhanced F_v/F_m was evident in the upper 25 m; and F_v/F_m was unchanged outside the enrichment area. c, 7 days after the initial iron addition, F_v/F_m was enhanced throughout the upper mixed layer (~50 m) within the enrichment area. Less than a day later, F_v/F_m began to slowly decrease back to pre-enrichment levels. Vertical distributions of F_v/F_m (a, b, c) were measured using a submersible fast-repetition-rate (FRR) fluorometer¹² mounted inside an undulating oceanographic recorder (UOR)³². The UOR was towed at night at <10 knots between ~5 and 70 m, with a horizontal undulation frequency (peak to peak) of ~1 km. The FRR exposes phytoplankton to a train of subsaturating blue-light flashes and measures the change in red light *in vivo* chlorophyll fluorescence from the initial dark-adapted state (F_0), when all functional photosystem II (PSII) reaction centres are oxidized, to the light-saturated state (F_m), when all PSII reaction centres have been photochemically reduced^{11,12}. Normalizing variable fluorescence ($F_v = F_m - F_0$) to F_m removes the effect of chlorophyll concentration on the absolute value of F_v , yielding a dimensionless, biomass-independent quantitative measure of the quantum efficiency of photochemistry. Each contour plot is based on between 1,000 and 3,400 individual measurements.

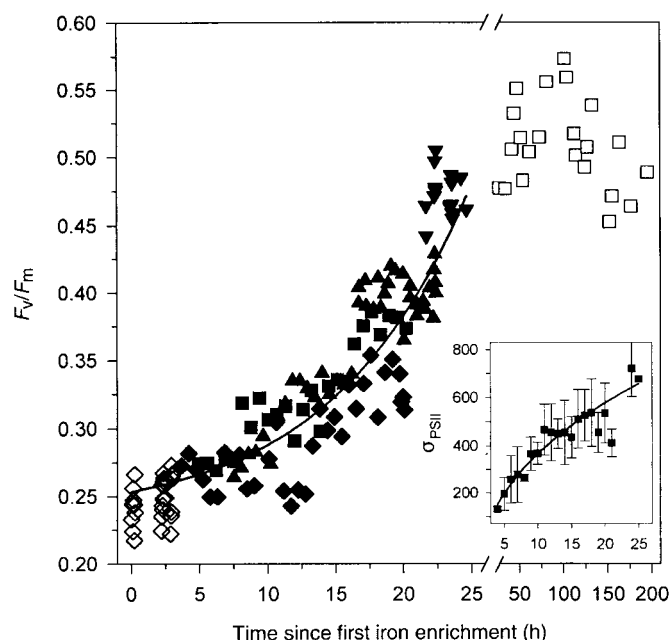


FIG. 2 Exponential increase in photochemical quantum efficiencies (F_v/F_m) of phytoplankton during the first 25 h following iron enrichment. The increase in F_v/F_m occurred within the extant phytoplankton assemblage dominated by *Prochlorococcus* sp. and *Synechococcus* sp.¹³. The initial 64 km² iron enrichment was added during 22 parallel 8-km transects, requiring ~16 h for completion⁸. High temporal resolution for the initial physiological response to iron enrichment was accomplished during three transects oriented perpendicular to the iron addition transects. Symbols $\blacklozenge$, $\blacksquare$, $\blacktriangle$ represent F_v/F_m measured at night during the first 3 transects through the enrichment area, respectively; $\blacktriangledown$, F_v/F_m measured during the following morning; $\diamond$, F_v/F_m measured outside the enrichment patch within 3.5 h of iron addition; $\square$, average F_v/F_m inside the enrichment area during the subsequent 200 h (8 d). Solid line is the best-fit ($r^2 = 0.86$) simple exponential model for data <25 h: $F_v/F_m = 0.02e^{0.1 \times \text{time(h)}} + 0.233$. Note break in the x-axis and change in scale between 25 and 50 h. Inset, changes in the functional absorption cross-section of photosystem II (σ_{PSII} in $\text{\AA}^2$ per photon) during the first 25 h following iron enrichment (mean $\pm$ s.d.). Measurement of F_v/F_m and σ_{PSII} were made using a benchtop fast-repetition-rate fluorometer¹² on a flow-through of sea water sampled from 3 m depth and dark-adapted for ~3 minutes.

that the severity of iron limitation in the phytoplankton assemblage was greater than during IronEx I (refs 4, 14). After the initial increase, σ_{PSII} remained between 750–950 $\text{\AA}^2$ per photon ($\lambda \approx 450$ –520 nm), while cellular chlorophyll concentrations continued to increase in all taxonomic fractions for ~ 7 d (ref. 13). The time course of change in F_v/F_m , σ_{PSII} and cellular chlorophyll indicates that phytoplankton responded to the relief of iron limitation first by rapidly enhancing their average photon processing potential per reaction centre, and then by increasing the cellular concentration of these photosynthetic units. Thus, the increase in photosynthetic capacity per cell was even greater than indicated by F_v/F_m alone.

Outside the enrichment region, σ_{PSII} remained low (mean $\pm$ s.d. = $230 \pm 80 \text{ \AA}^2$ per photon), F_v/F_m showed highly constrained diurnal maxima and nocturnal minima (Fig. 3), and surface NO_3^- remained largely unused⁸. Day-to-day physiological consistency in these unaltered populations throughout the study period suggests a highly evolved balance between iron availability and phytoplankton growth. The striking diel periodicity in photochemical quantum efficiencies (Fig. 3) appears to be a common feature of phytoplankton in the equatorial Pacific (unpublished data) resulting from nutrient-stressed growth conditions, as evidenced by the immediate loss of these cycles once iron limitation is relaxed (Fig. 3).

Results from the IronEx II experiment provide evidence critical to the long-standing debate about whether phytoplankton in the are incapable of fully utilizing available nutrients as a result of loss processes, such as zooplankton grazing, (that is, 'top-down' control)^{17–19} or because of micronutrient limitation (that is, 'bottom-up' control)^{1,20}. This debate has focused primarily on the time-dependent rate of change in phytoplankton biomass (dN/dt) observed *in situ* or during closed-bottle iron-enrichment experiments, which can be expressed by the ordinary differential equation $dN/dt = N(\mu - m)$, where N is the ensemble biomass of the phytoplankton community, μ is the average phytoplankton-specific growth rate, and m is the specific rate of mortality, which includes grazing. The equation can also be expressed for each individual species i , within the community²¹. Bottom-up control is defined as that condition where $\mu(i)$ is physiologically restricted to a value less than the relative maximum achievable specific growth rate for that species ($\hat{\mu}_{\text{max}}(i)$; ref. 22). As long as the condition $\mu(i) < \hat{\mu}_{\text{max}}(i)$ is satisfied, any change in N from growth or mortality results in an equivalent reciprocal change in μ , causing nutrient utilization to be independent of dN/dt . In contrast, top-down control is defined as that condition where N is sufficiently reduced by m to alleviate physiological constraints on $\mu(i)$, such that $\mu(i) \rightarrow \hat{\mu}_{\text{max}}(i)$. During top-down control, nutrient utilization is also independent of dN/dt at steady state (that is, $m = \hat{\mu}_{\text{max}}$; $dN/dt = 0$). It is therefore clear that bottom-up and top-down controls on nutrient utilization are mutually exclusive phenomena that cannot be distinguished through observations of dN/dt , but which by definition require measurements indicating the relationship between $\mu(i)$ and $\hat{\mu}_{\text{max}}(i)$.

Our results demonstrate a taxonomically independent physiological stimulation of phytoplankton following iron addition that corresponds to an increase in μ ²³ throughout the phytoplankton community. Such a response would not have occurred if nutrient utilization by the phytoplankton biomass was limited by loss processes. Thus, the root cause of the high-nutrient, low-chlorophyll (HNLC) phenomenon in the eastern equatorial Pacific Ocean is physiological limitation of phytoplankton by iron (bottom-up control). However, the physiological consistency and relatively stable biomass of the phytoplankton population outside the enrichment region¹³ indicate that $dN/dt \rightarrow 0$ as a result of $m \rightarrow \mu$.

Unlike terrestrial²⁴, intertidal²⁵ or lacustrine²⁶ systems, hypothesis-testing through *in situ* experimental manipulations resulting in alterations of community structure has never before been accomplished in the open ocean. The extended duration of IronEx II, compared to IronEx I, has provided unique insight

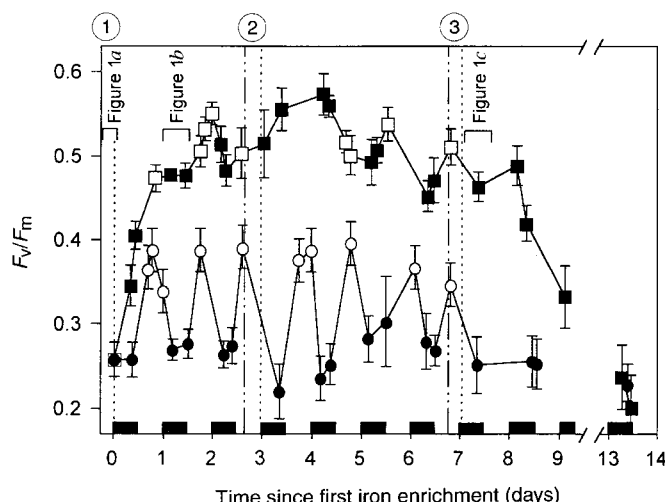


FIG. 3 Time course of change in F_v/F_m measured in water samples collected 3 m below the surface as the ship steamed during the first 13 d following iron enrichment. Squares, F_v/F_m (± 1 s.d.) inside iron-enrichment area; circles, F_v/F_m (± 1 s.d.) outside enrichment region. Open and solid symbols indicate data collected during daylight hours and at night, respectively; squares, average F_v/F_m for the iron-enrichment region and, thus, the initial increase in F_v/F_m after the first iron addition has a slightly different slope from that in Fig. 2. Dark and light spaces along the x-axis indicate successive day/night cycles. Numbered circles at the top of the graph indicate the time of the three iron additions. The UOR/FRR profile data used in Fig. 1 were collected during the three time periods indicated by the labelled brackets. Note the break in the x-axis between days 9 and 13.

into the evolution of a plankton community responding to an acute perturbation in iron availability. Our results document, with unparalleled temporal resolution, an immediate physiological stimulation of the natural picoplankton populations following the initial iron enrichment. Subsequent iron additions sustained elevated photosynthetic performance at comparable levels, despite taxonomic changes from a picoplankton- to diatom-dominated phytoplankton community¹³.

Although experimental and climatic timescales differ, iron-induced stimulation of the 'biological CO_2 pump' observed during IronEx II prompts speculation about the role of HNLC regions in regulating atmospheric CO_2 concentrations on glacial-interglacial timescales^{27,28}. However, model simulations¹⁰ and the lack of a clear relationship between terrigenous iron input and carbon sequestration in sediment core data from the eastern equatorial Pacific Ocean²⁹ indicate that chronic perturbations in the iron budget of this region are ineffective at significantly altering atmospheric CO_2 concentrations. The balance between phytoplankton growth rates and zooplankton grazing extant in the eastern equatorial Pacific Ocean suggests that, at timescales greater than the IronEx II experiment, increased iron availability can stimulate phytoplankton productivity and hence facilitate a drawdown of atmospheric CO_2 (ref. 30). In other HNLC regions, such as the southern Pacific Ocean, where seasonal variability in physical forcings are greater than in the eastern equatorial Pacific Ocean, long-term trophic responses may be disrupted and thus permit iron availability to have a greater influence on the 'biological CO_2 pump'¹⁰, as evidenced by sediment core data from these regions³¹. Insights gained during the IronEx experiments will prove invaluable in future studies assessing the biogeochemical importance of iron in these other HNLC regions. □

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CORRESPONDENCE should be addressed to M.J.B. (e-mail: mjb@warrior.das.bnl.gov).

Large decrease in ocean-surface CO₂ fugacity in response to *in situ* iron fertilization

D. J. Cooper*, A. J. Watson & P. D. Nightingale

Plymouth Marine Laboratory, Prospect Place, West Hoe, Plymouth PL1 3DH, UK and School of Environment Sciences, University of East Anglia, Norwich NR4 7TJ, UK

THE equatorial Pacific Ocean is a 'high-nitrate, low-chlorophyll' region where nitrate and phosphate are abundant all year round. These nutrients cannot therefore be limiting to phytoplankton production. It has been suggested that the bioavailability of iron—a micronutrient—may be preventing full biological utilization of the major nutrients^{1–3}. The results of a previous *in situ* iron fertilization experiment in this region provided support for this hypothesis⁴, but the observed biological response resulted in only a small decrease in surface-water CO₂ fugacity⁵. Here we report a much larger, biologically induced uptake of surface-water CO₂ that occurred during a second study⁶. The fugacity of CO₂ in the centre of the (iron-fertilized) patch of surface ocean fell from a background value near 510 μatm to approximately 420 μatm , corresponding to a transient 60% decrease in the natural ocean-to-atmosphere CO₂ flux. We conclude that iron supply to this ocean region can strongly modulate the local short-term source of CO₂ to the atmosphere, but has little long-term influence on atmospheric CO₂ partial pressure. However, if such a modulation also occurs in the Southern Ocean, then iron bioavailability at high southern latitudes could have a significant effect on atmospheric CO₂ partial pressure^{7–11}, for example over glacial–interglacial periods.

The equatorial Pacific and the Southern Ocean are the world's two largest high-nitrate low-chlorophyll (HNLC) regions.

Numerous incubation experiments support the possibility that iron limitation could contribute to this situation^{12–14}. To test the iron hypothesis under natural conditions, two enrichment experiments (IronEx I and II) have been conducted in the equatorial Pacific^{4,6}. These involved adding dissolved iron to a patch of surface water so as to attain nanomolar iron concentrations, and following the evolution of the subsequent biological and chemical signals. Despite subduction of the patch after three days, the analysis of IronEx I data demonstrated that iron addition had a pronounced effect on the ecosystem, with an initial several-fold increase in algal biomass, chlorophyll concentration and new production⁴. However, over the same period there was a minimal change in carbon dioxide fugacity (f_{CO_2}), suggesting only a minor role for iron in modulating the local ocean–atmosphere CO₂ flux⁵.

IronEx II (ref. 6) took place during May–June 1995 in a water mass starting at approximately 4° S, 104° W, which drifted to 7° S, 111° W over a three-week period of study. The water mass—with homogeneous physical, chemical and biological surface characteristics, including high nitrate concentrations (>10 nM), high f_{CO_2} (510 μatm , $\sigma = 3.5$, $n = 456$), and low biological indicators (biomass and chlorophyll)—was selected after an extensive survey. An 8 × 8 km patch was enriched by ferrous sulphate to a concentration of 2 nM and tagged with SF₆ as an inert tracer, using the technique described by Martin *et al.*⁴. This experiment differed from the IronEx I study in that two further 1 nM enrichments in ferrous sulphate were made three and seven days after the original infusion, to more closely mimic a natural, continuous supply of iron.

Measurements of f_{CO_2} in surface sea water were made using the ship's pumping system and an automated continuous, equilibrator/analyser¹⁵. Seawater f_{CO_2} measurements were recorded approximately every four minutes, with less frequent measurements of the atmospheric partial pressure of CO₂ (p_{CO_2}). The Li-Cor 6262 infrared detector was calibrated approximately hourly with gas standards interspersed between the sample measurements. A four-step procedure was employed to generate the *in situ* f_{CO_2} data: (1) preliminary CO₂ dry mole fractions were corrected by the Li-Cor software for band broadening and water-vapour interference, (2) true CO₂ dry mole fractions were

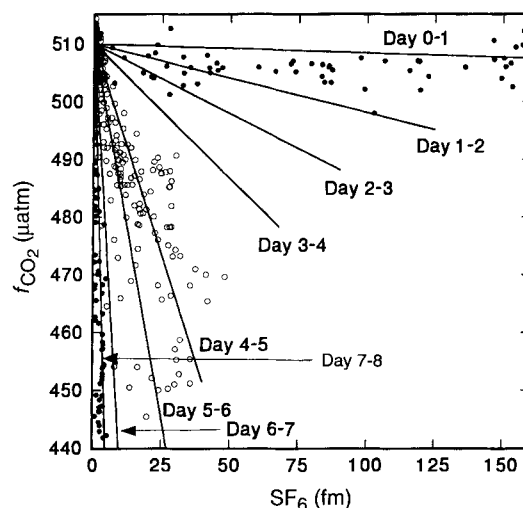


FIG. 1 Evolution of the f_{CO_2} drawdown in surface waters measured during IronEx II, plotted as a function of SF₆ concentration. Lines are least-squares linear regressions forced through the origin at the pre-infusion f_{CO_2} value of 510 μatm . The regression fits are calculated from data grouped into one-day intervals beginning immediately after the initial iron/SF₆ infusion. The higher concentrations of SF₆ for each line are 'in-patch', while near-zero concentrations are background values and represent control samples. Representative raw data are shown for days 0–1 (solid symbols), 4–5 (open symbols) and 7–8 (solid symbols).

* Present address: Department of Atmospheric and Oceanic Sciences, University of Wisconsin, 1225 West Dayton Street, Madison, Wisconsin 53706, USA.

calculated by correcting the preliminary values for detector drift, as determined from the standards, (3) equilibrator f_{CO_2} values were calculated by back-correcting the dry mole fraction to 100% humidity according to the saturated water vapour pressure at the equilibrator temperature and pressure¹⁵, and (4) a thermodynamic correction was applied to account for the temperature difference between the ship's sea inlet and the equilibrator¹⁶. The water in the equilibrator was $\sim 0.3^\circ\text{C}$ cooler than at the ship's seawater inlet, owing to travel through air-conditioned areas of the ship. This resulted in a correction of slightly more than +1% to the initially recorded f_{CO_2} value.

The SF_6 tracer data presented here were also measured underway from the ship's pumping system, using an automated gas chromatographic system with an electron capture detector^{17–19}. Time-integrated concentrations were recorded every three minutes. The initial tracer release of 0.5 moles (total) was sufficient to raise the ocean surface concentration in the patch by a factor of ~ 350 above background levels.

Figure 1 shows regression fits of *in situ* f_{CO_2} plotted against SF_6 concentration in surface water for the first 8 days of the experiment. During this time, f_{CO_2} declined rapidly at the centre of the patch as a consequence of the greatly stimulated phytoplankton growth^{6,20}. Over the same period, SF_6 concentrations at the centre of the patch declined by a factor of more than 20 due to areal spreading, deepening of the mixed layer and loss of $\sim 60\%$ of the original SF_6 to the atmosphere by gas exchange. The progressively greater slope of the regression fit with increasing time shows the emergence of correlation between the low f_{CO_2} values and the high tracer values, confirming that the maximum change in f_{CO_2} was associated with the iron addition²¹. The relatively large scatter of the raw data (Fig. 1) is due in large part to the rapidity of the biological response. Each one-day regression fit was calculated with data from a number of individual transects which often showed unique relationships between f_{CO_2} and SF_6 concentration.

An areal map of the patch, showing both CO_2 and SF_6 , is given in Fig. 2 for a composite period from days 4–6. Despite the apparent scatter and local inhomogeneities, which may be a consequence of the long period and the complicating re-infusion of iron into the core of the original patch (without SF_6 during day 3), there is a very striking coherence between the two maps. Little change occurred in the background control (outside the patch) over the course of the experiment (Table 1), so the evolution of CO_2 drawdown in the core of the patch cannot be attributed to natural variability. Table 1 shows that f_{CO_2} dropped to a minimum

TABLE 1 Evolution of the CO_2 drawdown during IronEx II

Time after primary iron release (d)	Mean f_{CO_2} outside patch (μatm (σ , n))	f_{CO_2} minimum in core of patch (μatm)	Integrated CO_2 drawdown (10^8 g C)
Pre-release	510.1 (3.9, 106)	NA	NA
0.0–2.0	507.1 (4.6, 185)	493.7	0.32
2.0–4.0	510.7 (4.8, 244)	477.3	0.43
4.0–6.0	508.6 (4.0, 110)	448.0	1.9
6.0–8.0	509.6 (1.9, 8)	416.9	5.0
8.0–9.4	506.2 (3.5, 114)	434.3*	6.5†
12.8–16.6	NA‡	425.2	28.5†

Outside-patch f_{CO_2} values were defined as outside the 2 fM SF_6 contour. Patch-centre f_{CO_2} values were calculated as the mean of the lowest 20 measurements during the time period. The integrated CO_2 drawdown was calculated from a matrix filled by interpolating the available data, and using the mean surface layer depth obtained from hydrographic (CTD) casts. NA, not available.

* Upper limit, centre of patch not fully mapped.

† Lower limit, patch not fully mapped.

‡ Insufficient data for calculation.

of $417 \mu\text{atm}$ in the core of the patch, a maximum perturbation of approximately $90 \mu\text{atm}$. Despite the stabilization of f_{CO_2} in the patch centre after approximately six days, the patch was deepening and spreading horizontally throughout the study. Integration of the f_{CO_2} drawdown shows that the net uptake of CO_2 in the patch was increasing even at the conclusion of the study, presumably as iron-enriched water mixed with high-nutrient water outside the core.

These results demonstrate that, at least in the conditions pertaining to the present experiment, the addition of iron to the waters of the equatorial Pacific Ocean can trigger phytoplankton growth of sufficient intensity to fix substantial amounts of CO_2 , and with it much of the normally unused macronutrients⁶. The resulting shift in surface-layer f_{CO_2} modulates the local ocean-atmosphere flux of CO_2 , which is driven by the difference in partial pressure across the air-sea interface (Δp_{CO_2}). In the present experiment, Δp_{CO_2} decreased by 60% from a pre-fertilization value of $150 \mu\text{atm}$ to $60 \mu\text{atm}$. The lesser effect observed during the IronEx I study⁵ indicates, however, that addition of iron does not by itself guarantee a large effect.

Two obvious differences between the experiments were the subduction event during IronEx I (refs 4 and 5) and the repeated additions of iron during IronEx II. The different outcomes of the two experiments were probably a consequence of at least one of these differences. The subduction event during IronEx I would have changed the intensity of light available in the fertilized patch,

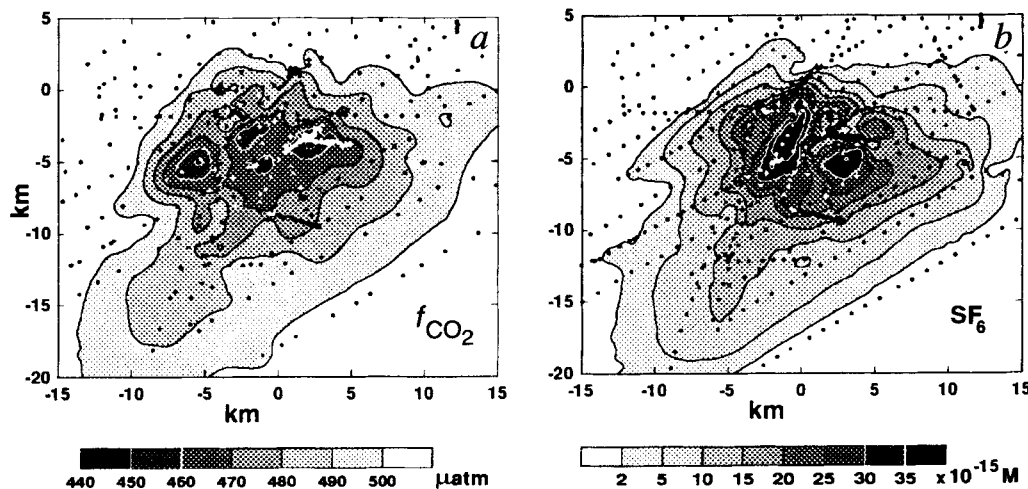


FIG. 2 The fertilized patch mapped in f_{CO_2} and SF_6 contoured using data obtained from 4–6 days after the initial iron/ SF_6 infusion. Sampling locations are indicated by solid symbols, corrected for the analytical time lag.

which could have limited the productivity. Repeated additions of iron during IronEx II were designed to increase the iron bioavailability, sustaining primary productivity in the event of chemical or physical losses. However, iron speciation measurements during IronEx II showed that iron was maintained in a complexed form by biologically derived chelators²², and was not chemically precipitated from the system. The subsequent iron additions may therefore have had little incremental effect on the bioavailable iron.

The high surface f_{CO_2} in the equatorial Pacific contributes an annual efflux to the atmosphere of 1–1.5 GtC (refs 23–25). It might be expected that a sustained increase in the supply of iron to this region, if it resulted in lowered surface f_{CO_2} , would have a substantial effect on atmospheric p_{CO_2} . Modelling studies have shown to the contrary, however, that the atmospheric partial pressure is insensitive to the biological activity in the equatorial Pacific. The model of Sarmiento and Orr⁷, for example, predicted a change of less than 3 μ atm in atmospheric p_{CO_2} after a century of complete utilization of surface macronutrient in the equatorial region. By contrast, when Southern Ocean productivity was stimulated by iron addition in their model, a decrease of approximately 70 μ atm in atmospheric p_{CO_2} was predicted. This represents a change in p_{CO_2} of ~10% over the no-fertilization case, because rising CO_2 emissions that follow the IPCC 'business as usual' scenario²⁶ double the atmospheric CO_2 burden over the modelled 100-year period. Other models have given substantially similar results concerning increased macro-nutrient utilization in the Southern Ocean, predicting decreases ranging from 6% to 21% in atmospheric p_{CO_2} (refs 8–11).

The lack of sensitivity of atmospheric p_{CO_2} to the degree of fertilization of the equatorial Pacific Ocean can be understood by considering the fate of excess nutrient and CO_2 upwelled at the Equator. This water resides only a few months in the upwelling zone before moving into the surface water of the subtropical region, where it acts as a source of nutrients for the biota. Fertilization of the equatorial ocean therefore causes an increased particulate export flux to the deep ocean from the equatorial upwelling, but cuts off an equivalent flow of carbon and nutrients to the subtropical gyres. This very quickly reduces the particulate export generated from the warm surface ocean by an equivalent amount. Thus changes in the equatorial zone are compensated by a change in conditions in the subtropical gyres. This contrasts with the situation when the Southern Ocean is fertilized. Increasing the particulate export flux there does not affect the particulate export in the rest of the ocean, because water from that region is not a source for warmer surface waters.

The results of the IronEx II study strongly suggest that iron supply to the equatorial region can influence local surface-water f_{CO_2} , but our understanding of the factors influencing atmospheric CO_2 suggests that this can have little long-term effect on atmospheric p_{CO_2} (<0.5% decrease)⁷. If the Southern Ocean can be similarly influenced, changes to iron supply there could lower the atmospheric CO_2 concentration by 6–21% (refs 7–11) based on models of enhanced nutrient utilization. Climate-induced changes in iron supply to this region might therefore have contributed, for example, to glacial–interglacial changes in atmospheric p_{CO_2} (refs 27, 28), and modulation of the iron source would alter the size of the present oceanic CO_2 sink. Although the IronEx II results cannot be uncritically extrapolated to the very different biological and physical conditions of the Southern Ocean²⁹, the demonstration of a strong effect in the equatorial Pacific increases the weight of more circumstantial evidence^{30,31} in favour of an important role for iron in Southern Ocean carbon cycling. □

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CORRESPONDENCE should be addressed to D.J.C. (e-mail: djcoope1@facstaff.wisc.edu).

Increased dimethyl sulphide concentrations in sea water from *in situ* iron enrichment

Suzanne M. Turner*, Philip D. Nightingale*†, Lucinda J. Spokes*, Malcolm I. Liddicoat† & Peter S. Liss*

* School of Environmental Sciences, University of East Anglia, Norwich NR4 7TJ, UK

† Plymouth Marine Laboratory, Prospect Place, West Hoe, Plymouth PL1 3DH, UK

THE concentrations of bioavailable iron in the surface waters of some ocean regions may indirectly modulate climate by controlling phytoplankton productivity and thus the amounts of carbon dioxide¹ and dimethyl sulphide (DMS) that are exchanged with the atmosphere. Oxidation of DMS is involved in the formation of atmospheric sulphate particles, which can exert a climate cooling effect² directly (by scattering and absorbing solar radiation), and indirectly (by affecting cloudiness and hence global albedo). But direct evidence supporting the hypothesis that DMS production in the ocean is affected by iron availability is lacking. Here we report changes in the concentrations of DMS in response to *in situ* iron-enrichment during two ecosystem-scale experiments designed to investigate the biological and chemical effects of iron fertilization of under-productive surface ocean waters^{3,4}. The first such experiment revealed a limited overall biological response³ and no significant changes in DMS concentrations, although the concentrations of its biochemical precursor doubled⁵. The second experiment, designed to better mimic the natural process of iron enrichment, elicited a much stronger biological response⁴, and DMS concentrations increased by a factor of 3.5. This result provides direct support for an important link in the iron–DMS–climate hypothesis.

The concentrations of DMS and its algal precursor, DMSPp (dimethylsulphoniopropionate), can vary considerably over small

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spatial scales⁶, so temporal changes can only be established if measurements are made in the same body of water. As water masses are subject to the influence of currents and wind-driven mixing, the tracer sulphur hexafluoride (SF_6) was released simultaneously with the initial iron (ferrous sulphate) additions, at the beginning of the field experiment. The iron-enriched patches, each with an area of $\sim 60 \text{ km}^2$, were roughly tracked using drifting buoys^{3,4} and characterized using SF_6 mapping techniques⁷ which enable quantification of vertical and lateral mixing rates⁸. Three

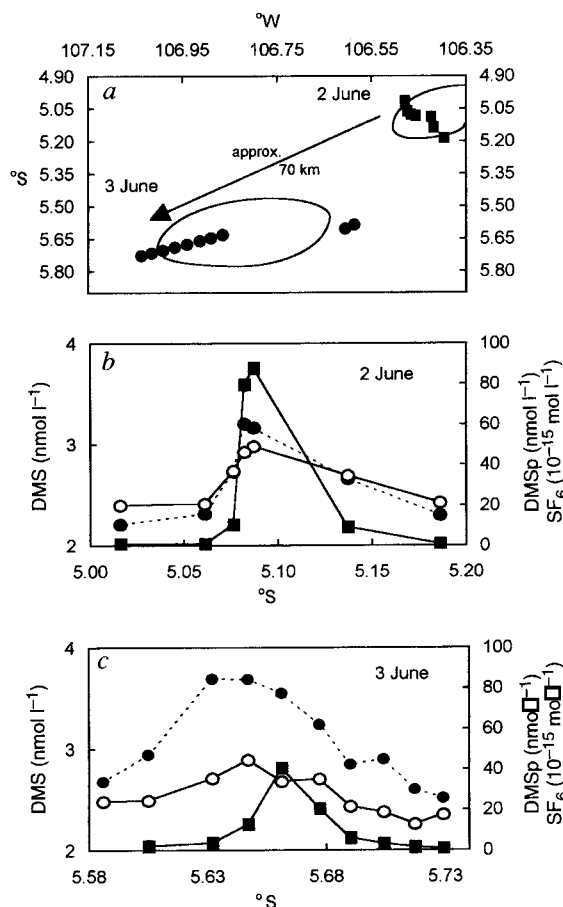


FIG. 1 Surface-water concentrations of DMSPP, DMS and SF_6 for two transects through patch 1, IronEx II, which was created on 29 May 1995 and initially covered an area of approximately $8 \times 8 \text{ km}$ in a fast-flowing northeast to southwest current. *a*, Sample positions across the patch on 2 June (squares) and 3 June (circles); ellipses indicate the areas with added iron and the arrow shows the direction and approximate distance travelled by the patch. *b*, Concentrations of DMS (filled circles), DMSPP (open circles) and SF_6 (open squares) at a depth of 15 m, 3.7 d after release. *c*, As *b* but at 3 m depth on day 5. Concentrations of the added tracer, SF_6 , indicate where iron had been added to the water. The end-members of each transect are considered to be outside the fertilized patch, as the low levels of SF_6 ($< 6 \times 10^{-16} \text{ mol l}^{-1}$) are consistent with surface water being in equilibrium with the atmospheric background of SF_6 . Mixing with this water caused dilution and spreading of the patch with time. The maximum levels of DMSPP on both transects were higher than concentrations found in background water (in patch, mean, 35.8 nmol l^{-1} ; range $18.8\text{--}49.2 \text{ nmol l}^{-1}$; $n = 11$; outside patch, mean 17.5 nmol l^{-1} ; range $12.9\text{--}31.2 \text{ nmol l}^{-1}$; $n = 34$). Maximum concentrations on the second transect were lower than on the preceding day. This may be due to spatial variability across the patch, but is more likely to arise from the difference in the depths of sampling, as DMSPP maxima were generally found between 15 and 20 m depth (see Fig. 2a). Concentrations of DMS were also elevated within the patch, relative to outside (in patch, mean, 3.1 nmol l^{-1} ; range $2.7\text{--}3.7$; $n = 11$; outside patch, mean, 2.4 nmol l^{-1} ; range $1.7\text{--}3.3 \text{ nmol l}^{-1}$; $n = 34$) and maximum levels were higher on 3 June than on the preceding day.

patches with different iron additions were studied. In the first experiment, IronEx I, a patch was enriched to $4 \text{ nmol l}^{-1} \text{ Fe}$ (ref. 3) ($\sim 500 \text{ km}$ south of the Galápagos Islands). For the second experiment, IronEx II, patch 1 was enriched with three additions of iron over 7 days to 2, 1 and $1 \text{ nmol l}^{-1} \text{ Fe}$, and patch 2 (corresponding to patch 3 in ref. 4) to $0.3 \text{ nmol l}^{-1} \text{ Fe}$ (ref. 4) (both patches $\sim 1,300 \text{ km}$ west of the Galápagos Islands). Concentrations of SF_6 decreased with time through mixing and dilution with the waters surrounding the patches and by emission to the air (Figs 1 and 2).

During IronEx II, DMSPP and DMS increased to concentrations which were well above the ranges observed for the natural background (Figs 1 and 2). The changes observed in the three patches were very similar over the first few days (Fig. 3), despite the order-of-magnitude difference in the initial iron concentrations (0.4 compared to $4.0 \text{ nmol l}^{-1} \text{ Fe}$). The increases in chlorophyll concentrations were very different; three-fold for IronEx I and about ten-fold for IronEx II, patch 1 (refs 3 and 4). It is recognised that particular groups of phytoplankton synthesize significantly more DMSPP than others⁹, so it is possible that during IronEx II the more prolific DMSPP-producing species constituted a smaller proportion of the increased biomass than on IronEx I. DMSPP concentrations increased immediately after enrichment and peaked on day 4 (Fig. 3a). The high concentration of DMSPP, found on day 13, suggests that declining concentrations resulted from mixing and dilution processes, rather than a decrease in production. DMS concentrations showed no significant increase during the first 2 days after enrichment (Fig. 3b), after which all samples (excluding IronEx I, because of subduction of the patch³) showed elevated concentrations. As DMS is considered to be generated by degradative processes such as cell lysis (caused by a virus or nutrient limitation and zooplankton grazing⁶), the time-lag between increases in DMSPP and DMS concentrations is to be expected.

We conclude that the addition of iron stimulated the synthesis of DMSPP and that this caused enhanced production of DMS. Although the changes in DMSPP concentrations observed in the three enrichment studies of IronEx I and II were very similar, the responses of the microbial communities were different in terms of biomass and DMS. Little is known about the spatial variation of ecosystems across high-nitrate low-chlorophyll (HNLC) regions, and the effects and frequency of changes in hydrography—such as the subduction of the patch on the third day of the IronEx I study—are uncertain. Further, although the experiments were ‘state of the art’, it was not possible to determine how the microbial communities would evolve over the longer term, in an environment where higher iron inputs would be expected to be more widespread and sustained. Therefore, extrapolation of the results to the oceanic scale is not straightforward. Nevertheless, increases in surface-water concentrations of DMS were observed and, as the magnitude of the sea-to-air flux depends on concentration and wind, we suggest that augmentation of aqueous iron concentrations will lead to increased emissions of sulphur to the atmosphere.

We now speculate on the possible implications of our findings for climate. The major source of new iron, and other trace nutrients, to open ocean surface waters is continentally derived aeolian dust^{9,10}; a study of an episodic dust event, north of Hawaii, reported¹¹ an increase in primary productivity by a factor of 1.6. Initial productivity increases occurred in the upper surface waters, then at greater depth, consistent with a supply of iron by dissolution from dust particles settling through the water column, rather than an input of nutrients from below, when the higher wind speeds increased the depth of the surface-ocean mixed layer¹¹. The authors suggested that the increased productivity ceased either because the aeolian iron supply became exhausted or because there was a transition from iron limitation to nitrate limitation. During IronEx I, the major nutrients were still at high concentrations when the increased productivity began to decline. This decline may, perhaps, have been due to loss of iron from

FIG. 2 Averaged depth profiles for SF_6 (a), DMSPp (b) and DMS (c) during the first 6 days of patch 1, IronEx II. The profiles of SF_6 (a) show that the tracer was well mixed in the top 20 m of the water column, that is, in the region above the thermocline. There was limited penetration of SF_6 below this depth and the decrease in surface-water concentrations with time was mainly due to lateral mixing with untreated water ($30\text{--}35\% \text{ d}^{-1}$), but also included a loss of between 5 and $12\% \text{ d}^{-1}$ via emission to the atmosphere⁴. The averaged profile at -1.3 days shows the background levels before tracer and iron deployment. Panel b shows that there was a subsurface maximum in DMSPp at ~ 20 m depth, and that concentrations at all depths increased with time. The profiles for DMS concentrations in the top 20 m (c) showed little variation for the first 2 days, but had doubled by day 5. Maximum DMS concentrations were situated below the maxima in DMSPp (b and c); there are two interpretations of these observations, as the analysis of DMSPp does not distinguish between viable phytoplankton cells, feeding zooplankton or detrital particles. First, that DMSPp was exported from the mixed layer and converted to DMS below. Second, that the effects of iron were not restricted to the mixed layer and stimulated growth at greater depths. It is likely that DMS production in the thermocline would have had little influence on the observed concentrations in the mixed layer as the SF_6 distributions indicated limited vertical diffusion. The deep production of DMS would only affect sea-to-air fluxes if there were an increase in the mixed layer depth, as occurs with an increase in wind speeds.

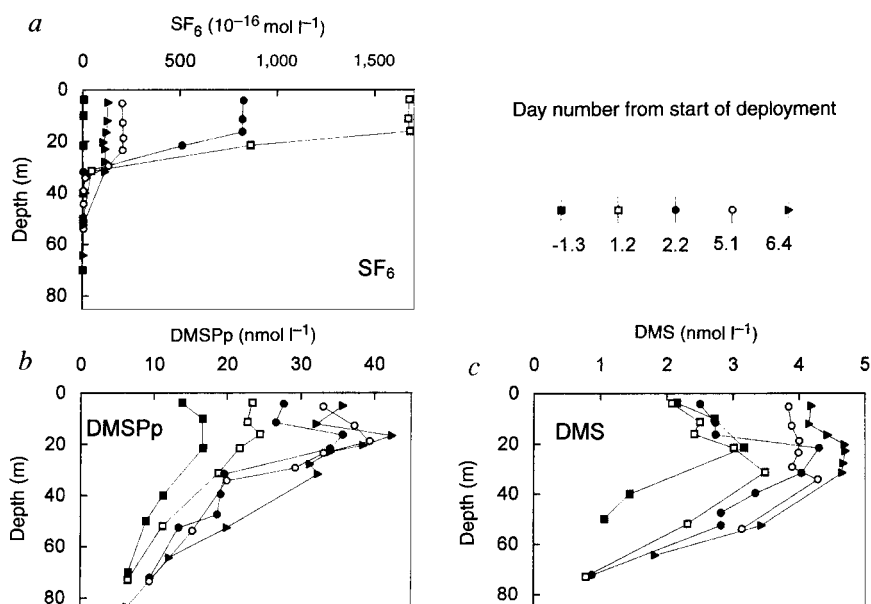
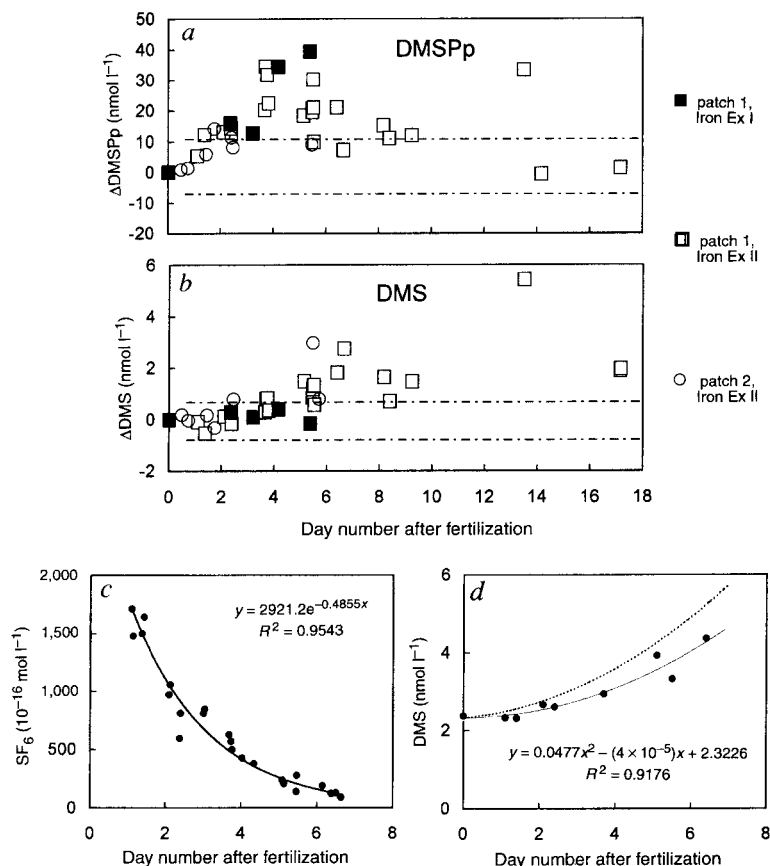


FIG. 3 Changes in DMS and DMSPp during the IronEx fertilization experiments (data shown for the IronEx I experiment are those before subduction of the patch²). Surface-water data (typically 5 m depth) for DMSPp (a) and DMS (b) from the three enrichment experiments are combined to give a single experimental time-course. IronEx I, patch 1 filled squares; IronEx II, patches 1 and 2, open squares and circles respectively. Concentrations have been normalized to the means of pre-enrichment data, time zero represents commencement of iron deployment. The means and ranges of DMS and DMSPp concentrations in untreated waters of the experiments were similar. Ironex I: DMS, 2.3 nmol l^{-1} ($1.9\text{--}3.1 \text{ nmol l}^{-1}$); DMSPp, 27.8 nmol l^{-1} ($7.5\text{--}34.3 \text{ nmol l}^{-1}$) and IronEx II: DMS, 2.4 nmol l^{-1} ($1.7\text{--}3.3 \text{ nmol l}^{-1}$); DMSPp, 17.5 nmol l^{-1} ($12.9\text{--}31.2 \text{ nmol l}^{-1}$). Two standard deviations of these data are shown as the dashed lines either side of the mean values at time zero. Statistical analysis (t-test) of the data collected each day after enrichment shows that DMSPp had increased significantly on day 1 with a 98% confidence limit, rising to 99% on day 3. No significant change was seen for DMS until day 3 (98%). Panels c and d show how concentrations (filled circles) of SF_6 (c) and DMS (d), averaged over the top 20 m of the water column, changed during the first 6 days of patch 1, IronEx II. Curve-fitting (solid lines and equations) provides estimates of the rate of dilution of the patch by water mixing and loss to the atmosphere (c) and the net increase in DMS in the surface layer (d). The dashed line in d gives an estimate of what the concentration of DMS would have been, without dilution by background levels.



surface waters (via particle formation and settling), increased rates of zooplankton grazing⁶, limitation by another trace nutrient or a decrease in the photodissolution of colloidal iron (III) when the patch was subducted²⁵. Further indications of the importance of iron to biological productivity comes from the Southern Ocean, where strong maxima in iron and phytoplankton concentrations, and CO₂ undersaturation, have been found at the Polar Front¹². Other work has shown that DMS concentrations are higher at Antarctic fronts¹³.

All these studies suggest that a climate with wind speeds higher than at present could sustain higher biological productivity. Concentrations of iron in surface waters would be enhanced by greater transport and deposition of dust and also by increased upwelling. Information about past climate is provided by the atmospheric record preserved in polar ice and the history of marine environments, recorded in deep ocean sediments. There is evidence that before the end of the last ice age (15 kyr before present), the atmosphere of the Southern Hemisphere contained elevated concentrations of iron¹⁴, methane sulphonic acid¹⁵ (MSA: an atmospheric oxidation product of, and specific to, DMS) and sulphate¹⁶, which accompanied low CO₂ concentrations¹⁴ (Fig. 4). Although there are sources of atmospheric sulphate other than oxidation of DMS and sea-salt sulphate, its covariation with MSA strongly suggests that the sulphate is primarily derived from biogenic, oceanic emissions¹⁶. This supports, but does not prove, the hypothesis that increased deposition of iron causes an increase in oceanic productivity, resulting in greater drawdown of CO₂ and enhanced atmospheric DMS concentrations.

Data for total organic carbon (TOC; Fig. 4b) in sediments of the

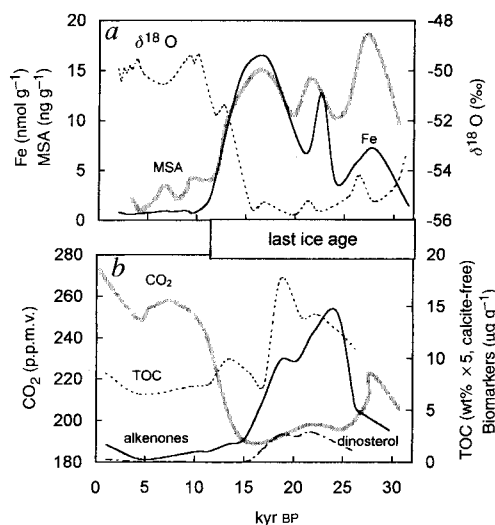


FIG. 4 Ice-core and sediment data from the literature for the Holocene and end of the last ice age. *a*, Methane sulphonic acid (MSA, a proxy for atmospheric DMS concentrations; grey line) and $\delta^{18}\text{O}$ (a proxy for temperature; dashed line) in an ice core from Dome C, east Antarctica (3,250 m elevation)¹⁵, estimated Fe concentration (solid black line) in an ice core from Vostok, Antarctica (aluminium data have been converted to Fe using crustal-abundance values)¹⁴. *b*, CO₂ concentration (parts per million by volume, p.p.m.v.; grey line) in an ice core from Vostok, Antarctica¹⁶, total organic carbon (TOC, proxy for surface ocean productivity; dashed line), alkenones (solid black line) and dinosterol (dot-dashed black line) in a sediment core from the eastern tropical Pacific (0°57.2'N, 138°57.3'W)¹⁷. The biomarkers, dinosterol and alkenones, are derived from dinoflagellates and prymnesiophytes, respectively; these groups of phytoplankton are known to be associated with significant DMS production. Dating of each of the records is by $\delta^{18}\text{O}$ measurements (trapped air in ice cores and the foraminifera *Globobulimina tumida* in the sediment core), which show only minor differences. The sites of both ice cores are at high elevations and the data are considered to represent the global, rather than local, atmosphere¹⁶.

tropical Pacific¹⁷ also suggest that productivity in the upper water column was enhanced during the last ice age. The increased concentrations of biomarkers¹⁷, derived from the species of phytoplankton which currently are known to be significant DMSpp producers¹⁸ (Fig. 4b) further suggest that there may have been enhanced DMS production. However, we cannot necessarily assume that the trends observed for the Antarctic and Pacific were coupled, representing a unified global trend, for there may have been different, localized perturbations. Indeed, an ice record from the Arctic shows that MSA concentrations were lower during glacial periods than in warmer climates, the converse of the findings from Antarctica¹⁹. Nonetheless, atmospheric sulphate concentrations of the Southern Hemisphere were elevated at the end of the last ice age, which current knowledge suggests would have had significant implications for climate.

Sulphate aerosols are thought to exert, after greenhouse gases, the next largest influence the atmosphere, by bringing about both absorption and scattering of solar radiation (direct effect) and through changes in the number density of cloud condensation nuclei (CCN) and hence cloudiness (indirect effect). For example, it has been calculated² that for the indirect effect, a 30% increase in CCN number density leads to a global cooling of 1.3 K. For the direct effect, a cooling of 0.6 K is predicted²⁰ if sulphur emissions were to increase by 70% (ref. 21). Other work has shown that there may be considerable variation in the degree of cooling, depending on geographical region, because the relationship between CCN number density and reflectance is nonlinear²². For example, in environments such as remote oceanic areas, where the atmospheric particle loading is low, a large change in reflectance can be produced by only small changes in particle density. Thus, the albedo of the Antarctic region over both ocean and ice would be particularly sensitive to increases in atmospheric sulphate²². Further, as the Antarctic oceans are also classified as HNLC regions, they may be particularly sensitive to increases in iron availability.

On the basis of the results of the IronEx experiments, we suggest that iron enrichment tends to increase the emission of DMS and that this could account for the increased concentrations of Antarctic aerosol sulphate found during the last glacial period. We further speculate that enhancement of biologically available iron may lead to a significant cooling effect via DMS-derived CCN.

Results from the IronEx experiments indicate, at least for the equatorial Pacific region, that changes in DMS production are likely to be climatically more significant than iron-induced changes in the uptake of CO₂ by the ocean^{23,24}. Clearly, there is an urgent need to test these ideas in the Southern Ocean, where both enhanced uptake of CO₂ (ref. 24) and production of DMS are likely to be important. □

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CORRESPONDENCE should be addressed to S.M.T. (e-mail: s.turner@uea.ac.uk).

The osmium isotopic composition of the Earth's primitive upper mantle

T. Meisel*[‡], R. J. Walker* & J. W. Morgan[†]

* Isotope Geochemistry Laboratory, Department of Geology, University of Maryland, College Park, Maryland 20742, USA

[†] Department of Earth Resources, Colorado State University, Fort Collins, Colorado 80523, USA

THE elevated abundances of highly siderophile elements in the Earth's mantle, relative to what would be predicted from metal-silicate equilibrium, have often been cited as evidence for the accretion to the Earth of a 'late veneer' of chondritic material following core formation¹. As rhenium and its decay-product osmium are both highly siderophile, the evolution of the Re–Os isotope system in a terrestrial reservoir provides a robust, time-averaged constraint on the siderophile abundances of the reservoir; thus, the broadly chondritic evolution of Os isotopes in the oceanic upper mantle provides strong support for the late accretion model^{2,3}. But the Re–Os composition of the late veneer is still poorly defined, because the mantle has differentiated into ¹⁸⁷Os-enriched and -depleted reservoirs^{4–7}. Here we report a value for the Os isotopic composition of the modern 'primitive upper mantle' (PUM), a hypothetical undifferentiated upper-mantle reservoir. From suites of variably melt-depleted mantle xenoliths from three continents, we derive a minimum ¹⁸⁷Os/¹⁸⁸Os ratio for PUM of 0.1290 ± 0.0009 , by using a correlation between ¹⁸⁷Os/¹⁸⁸Os and geochemical indices of 'fertility' to extrapolate to the Os isotope ratio of undepleted mantle. Comparing this value to the ¹⁸⁷Os/¹⁸⁸Os ratios measured in different classes of chondritic meteorite, we infer that the late veneer had siderophile element abundances similar to those of enstatite or ordinary chondrites (¹⁸⁷Os/¹⁸⁸Os = 0.1286 ± 0.0010), rather than carbonaceous chondrites (0.1258 ± 0.0005).

The Os isotopic composition of modern mantle reservoirs is heterogeneous. Rhenium is a mildly incompatible element during mantle melting, so melt depletion reduces the Re/Os of mantle residues⁴. In contrast, the injection of Re-rich basaltic melt or crust into a mantle domain increases the Re/Os of the mix, which then evolves to suprachondritic ¹⁸⁷Os/¹⁸⁸Os (ref. 6). The isotopic composition of Os in the hypothetical modern bulk upper mantle, historically referred to as PUM^{8,9}, is the composition that would have evolved from the late accretionary period (ending ~3.9 Gyr ago) to the present had there been no modification of the Re/Os by melt depletion or enrichment. If the upper and lower mantle are homogeneous with respect to highly siderophile elements (HSE), then the Os isotopic composition of PUM is also equivalent to the composition of the bulk silicate Earth (BSE).

The Os isotopic composition of PUM directly reflects the

relative abundances of HSE added to the Earth by late accretion. In addition, the Os isotopic composition of PUM can constrain what, if any, long-term Re depletion the depleted mid-ocean-ridge basalt mantle (DMM) has suffered relative to PUM. Previous studies of ¹⁸⁷Os-enriched and -depleted materials assume that they were ultimately derived from mantle whose Os isotopic composition evolved linearly from a meteoritic initial composition to the modern average value measured in abyssal peridotites (similar to that of carbonaceous chondrites)^{2,3,10,11}. Interpretations resulting from such models may not be valid, however, if the composition of PUM is not equivalent to that of DMM.

It is unlikely that any pristine primitive mantle materials exist today, so the Os isotopic composition of PUM cannot be determined directly. Our approach is to determine the Os isotopic composition of the most fertile endmember of variably melt-depleted mantle xenolith suites. Previous studies of individual suites of xenoliths and orogenic peridotites^{4,12,13} have shown good positive linear correlations between ¹⁸⁷Os/¹⁸⁸Os and indices of fertility, such as Al_2O_3 and $\text{Mg}/(\text{Mg} + \text{Fe}^{2+})$. Consequently, there is strong evidence that the Re/Os ratio of residues resulting from mantle melting decrease gradually and linearly as the extent of melting increases. In these studies, data for the most melt-depleted xenoliths have been used to provide constraints on when isolation from the convecting mantle occurred. Conversely, those xenoliths within a suite that show no evidence for significant melt depletion (or enrichment) may retain a fertile Re/Os and continue to grow ¹⁸⁷Os at a rate consistent with the Re/Os of PUM. Their present day composition may provide a minimum estimate of PUM. For suites resulting from variable extents of melt depletion, ¹⁸⁷Os/¹⁸⁸Os data can be plotted versus an index of fertility and linearly regressed. The intersection between the resulting line and the estimated concentration of the index element in PUM provides the ¹⁸⁷Os/¹⁸⁸Os for the undepleted end-member.

For this study, Al_2O_3 and Lu contents are used as indices of fertility. The average estimated PUM values used for Al_2O_3 and Lu are 4.2 weight per cent and 68 ng g^{-1} , respectively^{14,15}. A direct correlation between Al_2O_3 and ¹⁸⁷Os/¹⁸⁸Os can, however, be hampered by variations in the abundances of minerals that are not influenced by melt removal. For example, varying amounts of spinel can cause scatter in the Al_2O_3 concentration of a rock without necessarily reflecting extent of melt removal. Because the abundance of Os is primarily controlled by intergranular sulphides^{16,17}, variations in clinopyroxene and spinel abundance do not affect the isotopic composition. Trace element indices of fertility are therefore also useful in such comparisons. The trace element Lu is useful for comparison with ¹⁸⁷Os/¹⁸⁸Os because, during mantle melting, Lu and Re are similarly incompatible¹⁸. Unlike the light rare-earth elements (LREE), Lu may be little affected by metasomatic processes.

We examined (Table 1): (1) a suite of seven spinel-peridotite xenoliths from the Kilbourne Hole volcanic field (Rio Grande Rift, SW USA)^{19,20}; (2) three spinel-peridotite xenoliths from the Dreiser Weiher field (Westefel, Germany)²¹; and (3) one highly fertile spinel-peridotite from the Tariat depression (Baikal rift, Mongolia)²². To avoid misinterpretation due to secondary changes in the mineralogical-chemical composition, only coarse-grained, protogranular spinel-peridotite xenoliths from each suite were studied. All three of these volcanic fields are situated in extensional continental settings.

There are high linear correlations between ¹⁸⁷Os/¹⁸⁸Os and Lu for Kilbourne Hole and Dreiser Weiher suites (Fig. 1). Correlations between ¹⁸⁷Os/¹⁸⁸Os and Sc for both suites (not shown) are quite similar. The correlation between ¹⁸⁷Os/¹⁸⁸Os and Al_2O_3 is good for the Dreiser Weiher suite but somewhat scattered for Kilbourne Hole. The most fertile Os isotopic compositions determined for both suites using both Al_2O_3 and Lu correlations are very similar, with the intersections between the regression lines and average estimates for PUM ranging from 0.1281 to 0.1293. The single sample from Tariat has Al_2O_3 and Lu concentrations

[‡] Present address: General and Analytical Chemistry, University of Leoben, Franz-Josef-Str. 18, A-8700 Leoben, Austria.

TABLE 1 Isotopic and composition data for mantle xenoliths

		Lu ($\mu\text{g g}^{-1}$)	Al ₂ O ₃ (wt %)	Sc ($\mu\text{g g}^{-1}$)	Re (ng g ⁻¹)	Os (ng g ⁻¹)	¹⁸⁷ Re/ ¹⁸⁸ Os	¹⁸⁷ Os/ ¹⁸⁸ Os	ϵ_{Nd}
Kilbourne Hole	UM-2	0.054	3.36	13.8	0.253	3.11	0.393	0.1254	+9.4
	UM-2				0.251	3.13	0.387	0.1256	
	UM-3				0.118	2.48	0.228	0.1259	
	UM-3					2.49		0.1259	
	UM-4	0.061	3.57	14.5	0.084	1.58	0.253	0.1274	
	UM-4					1.60		0.1269	
	UM-6	0.064	4.07	16.0	0.176	2.77	0.306	0.1268	
	UM-9	0.060	3.19	15.0	0.432	3.27	0.635	0.1271	
	KH77-1	0.078	4.29	17.1	0.233	2.88	0.377	0.1290	
	KH77-14	0.062	3.47	14.9	0.213	2.30	0.446	0.1298	
Dreiser Weiher	KH77-14				0.211	2.20	0.463	0.1297	
	Ib/2	0.008	0.64	4.3	0.006	1.62	0.018	0.1180	
	Ib/3	0.022	2.11	10.2	0.069	2.70	0.127	0.1224	+1.6
Tariat	Ib/8	0.077	4.29	18.3	0.115	2.37	0.232	0.1297	+10.0
	MHP79/2	0.073	4.34	14.1	0.119	2.74	0.209	0.1283	+10.3

Sources of data: Lu, Al₂O₃, Sc, and Nd isotopic composition^{19–23}. Lu data for Dreiser Weiher were obtained by RNAA all other trace element data by INAA. Precision of analysis Al₂O₃ (3%), Lu and Sc (2–10%). Whole-rock sample powders were digested with Carius tubes³⁵ and analysed for Re–Os by negative thermal ionization mass spectrometry using procedures described previously³⁶. Replicates are separate dissolutions on the same or different sample split. Errors on ¹⁸⁷Re/¹⁸⁸Os and ¹⁸⁷Os/¹⁸⁸Os are estimated at 5 and 0.2% respectively. Re and Os blank corrections were applied for all samples. Total analytical blanks were 35 ± 10 pg Re and 11 pg Os with an ¹⁸⁷Os/¹⁸⁸Os of 0.173.

that are very close to the average values for PUM (Table 1). It has an ¹⁸⁷Os/¹⁸⁸Os within this range (0.1283). In addition to these data, the linear regressions of ¹⁸⁷Os/¹⁸⁸Os versus Al₂O₃ previously reported for Ronda and eastern Pyrenean peridotite massifs^{12,13} give similar ¹⁸⁷Os/¹⁸⁸Os ratios of 0.1281 and 0.1301, respectively, at their intersections with the average PUM estimate for Al₂O₃. Combining the data for the five suites yields an average ¹⁸⁷Os/¹⁸⁸Os of 0.1290 ± 0.009 (1 standard deviation), consistent with a ¹⁸⁷Re/¹⁸⁸Os for PUM of 0.428. The minimal variation in Os isotopic composition indicates that the Re/Os evolution of these materials was quite similar.

Although similar ¹⁸⁷Os/¹⁸⁸Os are obtained for the fertile end-members of the different suites, no samples examined by this study have major- and trace-element characteristics wholly consistent with pristine PUM. For example, all the xenoliths examined in this study have Nd isotopic compositions similar to DMM, consistent with long-term LREE depletion^{20,22,23} (Table 1). The Nd isotopic compositions of these suites require that even the highly fertile xenoliths represent mantle that has been melted at some previous stage. Their fertile nature, therefore, indicates that they experienced either such a minor extent of melt extraction that major elements were little affected, or a multistage history in which melt depletion occurred, but the mantle domain was then subsequently

re-enriched in a basaltic component. In the former instance, the Re/Os likely would be only slightly reduced because Re is only mildly incompatible. In the latter instance, the addition of a basalt component into a previously depleted mantle domain may partially return Re abundances to their pre-depletion concentrations. In either event our PUM value must be considered a minimum.

As has been noted in previous studies of mantle xenoliths⁴ and orogenic peridotites¹³, Re abundances and ¹⁸⁷Re/¹⁸⁸Os do not correlate well with ¹⁸⁷Os/¹⁸⁸Os within each suite. For those samples with supra-chondritic ¹⁸⁷Re/¹⁸⁸Os, this may result from variable addition of Re from host basalts during ascent. Basalts typically have much higher concentrations of Re than peridotite xenoliths. This is in contrast to Os whose concentrations in mantle xenoliths is typically much higher than in basalts. Some fertile samples (Ib/8, MHP79/2), however, have ¹⁸⁷Re/¹⁸⁸Os lower than chondritic and, therefore, have unsupported ¹⁸⁷Os. This may reflect Re removal from the mantle source through an as yet unidentified process just before removal of the xenolith to the surface. It may also indicate selective removal of Re by means of the weathering of host phases at the surface.

To compare the value of PUM with possible late accretionary precursors, we analysed suites of ordinary, enstatite and carbonaceous chondrites (Table 2). Despite the problems of obtaining

FIG. 1 a, ¹⁸⁷Os/¹⁸⁸Os vs. Lu for Kilbourne Hole (circles), Dreiser Weiher (triangles), Tariat (square), and b, ¹⁸⁷Os/¹⁸⁸Os versus Al₂O₃ for the same xenolith samples, including samples from the eastern Pyrenean peridotite massifs¹³ (diamonds). The concentration range for Lu and Al₂O₃ in PUM (primitive upper mantle) is defined by the highest and lowest estimates from refs 8, 9, 14 and 15. Two samples lie outside the PUM field for Lu but would lie in the PUM field for Yb. Sample KH77-14 does not follow the linear trend observed for the other Kilbourne Hole samples for ¹⁸⁷Os/¹⁸⁸Os versus Lu. This can be explained by varying proportions of clinopyroxene, which does not affect the Os isotopic composition. The scatter for Kilbourne Hole samples in the ¹⁸⁷Os/¹⁸⁸Os versus Al₂O₃ plot can be explained by varying proportions of spinel. Linear regressions are plotted for Kilbourne Hole xenoliths (dashed) excluding KH77-14, Dreiser Weiher xenoliths (solid) and eastern Pyrenean orogenic massif peridotites (dotted) excluding the one odd sample. The PUM value for ¹⁸⁷Os/¹⁸⁸Os is based on the intercept of the regression with an average PUM value of Al₂O₃ 4.2 weight per cent and Lu 68 ng g⁻¹ (refs 14, 15). DMM range from ref. 26.

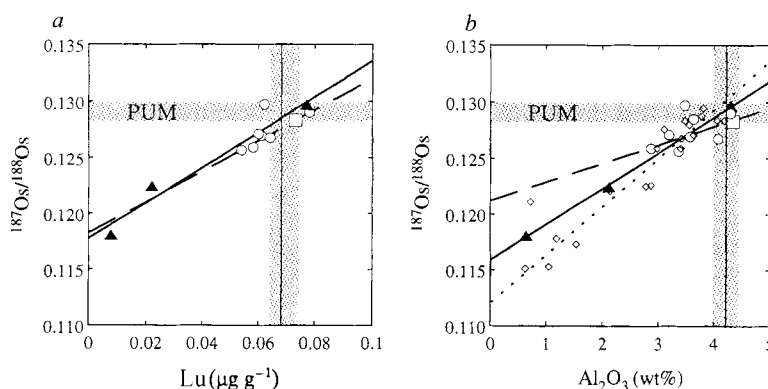


TABLE 2 Re and Os concentration and Os isotope composition data for chondrites

		Re (ng g ⁻¹)	Os (ng g ⁻¹)	¹⁸⁷ Re/ ¹⁸⁸ Os	¹⁸⁷ Os/ ¹⁸⁸ Os
Carbonaceous chondrites					
CM2	Al Raiz	42.30	546.1	0.3731	0.12543
CM2	Murray	54.13	672.4	0.3878	0.12559
CM2	Mighei*	49.05	593.2	0.3982	0.12631
CV3	Allende	63.23	773.9	0.3935	0.12610
	Allende	62.58	770.1	0.3915	0.12643
Ordinary chondrites					
H3	Dhajala*	73.91	816.9	0.4361	0.12853
H3	Sharps*	74.52	876.1	0.4099	0.12721
H3	Bremervörde	51.83	583.5	0.4281	0.12889
H4	Avanhandava*	78.75	842.7	0.4505	0.13045
H4	Forest Vale	80.67	824.3	0.4717	0.12926
	Forest Vale	78.78	794.4	0.4779	0.12972
H5	Anlong*	96.96	1048	0.4458	0.13033
H5	Changde*	44.66	498.2	0.4321	0.12853
H5	Allegan*	79.33	888.5	0.4303	0.12961
	Allegan*	76.72	860.2	0.4298	0.12877
H5	Forest City*	78.64	862.8	0.4393	0.12841
	Forest City*	69.60	802.3	0.4180	0.12649
H6	Guareña*	84.17	982.5	0.4129	0.12817
H6	Xingyang*	86.21	976.7	0.4255	0.13021
LL3	Chainpur*	35.76	417.9	0.4122	0.12793
Enstatite chondrites					
Eh3	Kota Kota	56.97	637.3	0.4307	0.12850
Eh4	Adhi Kot	56.96	648.2	0.4235	0.12833
Eh4	Indarch	49.42	573.3	0.4154	0.12809
Ei6	Atlanta	81.08	843.6	0.4632	0.12900
Ei6	Daniel's Kuil	88.05	998.9	0.4248	0.12853
Ei6	Hvittis	44.69	538.4	0.3999	0.12848
Ei6	Jai deh Kot Lalu	46.77	543.5	0.4147	0.12813
Ei6	Khairpur	72.73	823.2	0.4257	0.12857
Ei6	Pillistfer*	52.41	607.5	0.4156	0.12817
Ei6	Yilmia	78.81	940.5	0.4037	0.12723

0.1–0.2 g sample powder were digested with either the Carius tubes method³⁵ or by alkaline fusion³⁶ (asterisks). All samples digested and equilibrated using the Carius tube method were spiked using the mixed Re–Os spike discussed in ref. 37. Replicate analysis have Re/Os ratios that are not as reproducible as for iron meteorites³⁷. The minor variability in Re/Os cannot solely be the result of primary heterogeneities in the materials, because Os isotopic ratios generally do reproduce well. We believe these effects are the result of late-stage, open-system behaviour. These data will be discussed in greater detail in a future publication. Uncertainties are 0.3% for ¹⁸⁷Re/¹⁸⁸Os, and 0.05% for ¹⁸⁷Os/¹⁸⁸Os, 0.2 and 0.1% for Re and Os concentrations, respectively.

reproducible Re/Os for chondrites (see Table 2 legend), ¹⁸⁷Os/¹⁸⁸Os ratios are reproducible (generally to within ±0.5%), suggesting that the present day 'whole-rock' Os isotopic compositions of chondrites likely reflect the time-integrated compositions of the true Re/Os of each meteorite. For example, a suite of data for 12 ordinary chondrites define a narrow range of present day ¹⁸⁷Os/¹⁸⁸Os of 0.1289 ± 0.0011 (Fig. 2). Data for 10 enstatite chondrites define a similar average—present day ¹⁸⁷Os/¹⁸⁸Os of 0.1283 ± 0.0005. In contrast, a suite of four carbonaceous chondrites define a 1–2% lower ¹⁸⁷Os/¹⁸⁸Os of 0.1259 ± 0.0005 (Fig. 2). The Os isotopic composition difference between enstatite/ordinary and carbonaceous chondrites is consistent with previously recognized differences in Re/Os (refs 24, 25).

Our estimated Os isotopic composition for PUM is significantly higher than the average composition of carbonaceous chondrites, but similar to the average compositions of ordinary and enstatite chondrites. Although the average composition of DMM is not yet firmly established, studies of abyssal peridotites indicate an average ¹⁸⁷Os/¹⁸⁸Os of 0.1246 ± 0.0014 (ref. 26)—about 1% less than the average for carbonaceous chondrites and about 4% less radiogenic than PUM. This comparison suggests that melt removal from DMM has affected not only the highly lithophile elements, but also the Re budget of the largest upper-mantle reservoir. If abyssal peridotites accurately reflect the Os isotopic ratio of DMM, then at least a 7% removal of Re from PUM is required to explain the 4% lower ¹⁸⁷Os/¹⁸⁸Os ratio, even if all Re was removed from the upper mantle very soon after late accretion ceased 3.9 Gyr ago.

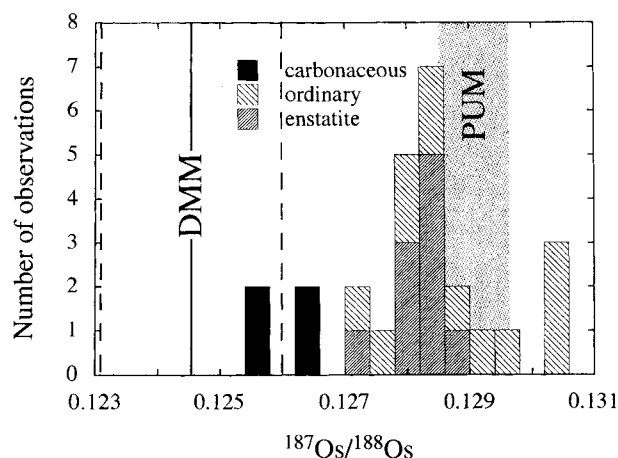


FIG. 2 Range of all chondrites measured with negative thermal ionization mass spectrometry. The primitive mantle average (PUM) is similar to enstatite and ordinary chondrites and distinctly different from carbonaceous chondrites. Range of depleted mid-ocean-ridge basalt mantle (DMM), the source for the oceanic crust, is marked by dashed lines and has a lower isotopic composition than PUM. This is explained by long-term Re depletion.

The chondritic Os isotopic composition of PUM provides additional support for the late accretion model, and is consistent with the conclusions of previous Os isotope studies of the upper mantle^{2,3}. Several recent studies have projected that metal-silicate distributions for HSE at high temperatures and pressures are substantially less than those determined in laboratory experiments at low temperature and pressure^{27,28}. These studies have speculated that the higher than expected abundances of HSE in the upper mantle may therefore reflect high-temperature and -pressure processing of HSE during core formation, and may obviate the need for the late accretion model. Our estimated PUM composition, however, requires that Re and Os were not fractionated from one another during the formation of PUM by more than approximately $\pm 2\%$. As the distribution coefficients for Re and Os are unlikely to be virtually identical²⁹, a late veneer seems to be required.

There has been little agreement regarding the best meteorite analogue for the composition of the late veneer; for example, carbonaceous chondrites have often been invoked to explain the abundances of volatile elements in the mantle^{30–33}, but H-chondrites provide a better match for sulphur³⁴. Our results are inconsistent with carbonaceous chondrites as the dominant contribution to the HSE budget of the late veneer, and suggest that ordinary and enstatite chondrites provide a better starting point for modelling the relative abundances of highly siderophile elements in the upper mantle. □

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CORRESPONDENCE should be addressed to M.T. (e-mail: meisel@unileoben.ac.at).

Hidden colour aversions in domestic chicks triggered by pyrazine odours of insect warning displays

Candy Rowe & Tim Guilford

Department of Zoology, South Parks Road, Oxford, OX1 3PS, UK

ANIMAL signals are often 'multicomponent'^{1–3}, consisting of displays with several parts to allow exploitation of different senses. An example is the courtship display of the peacock, which combines dashing movements with a noisy, shimmering show of his train feathers⁴. Yet the significance of multicomponent signalling is unknown. As a model multicomponent signalling system, we investigated the warning signals of toxic insects, which often combine pyrazine odours with conspicuous coloration such as yellow or red^{5–7}. Here we demonstrate, in prey choice experiments with birds, that pyrazine interacts with red and yellow to induce strong aversions to these aposematic colours that are not shown in the absence of the odour. Pyrazine is shown neither to be inherently aversive, nor to induce aversion to a non-aposematic colour, green. To our knowledge, this study provides the first evidence that the function of multicomponent signals can lie in hidden psychological responses produced by the interaction of their components.

To date, the function of warning colours^{8,9} and warning odours such as pyrazine⁶, have usually been investigated in isolation. As pyrazine odours are often perceived in combination with conspicuous coloration as part of multicomponent warning displays^{5–7,10}, we aimed to determine the significance of their interaction. Warning colour function was simulated by investigating how domestic chicks (*Gallus gallus domesticus*) performed in an artificial prey-discrimination task in which colour cues signalled the palatability of food items. The interactive effect of pyrazine on warning-colour function was determined by presenting this

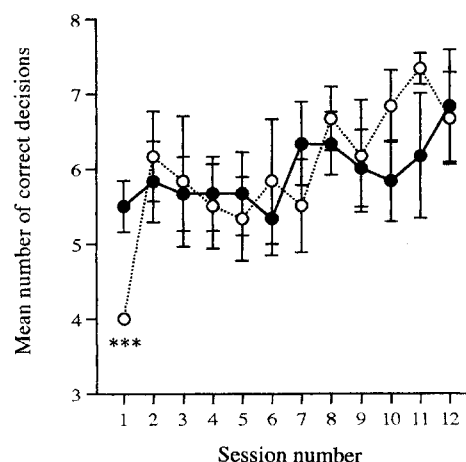


FIG. 1 The result of experiment one plotted as mean number of correct decisions ($\pm$ s.e.m.) per session for each treatment group of six birds. A correct decision is defined as eating a palatable crumb or ignoring an unpalatable crumb, so that 4 correct decisions per session indicates no discrimination, whereas 8 indicates completely correct discrimination, 0 indicates completely incorrect discrimination. Six birds in each treatment group (pyrazine present and pyrazine absent) received 12 sessions of 8 prey encounters, sessions spaced by about 30 min. Open circles: yellow unpalatable prey, pyrazine absent; Filled circles: yellow unpalatable prey, pyrazine present. ***, $P < 0.001$.

discrimination task either in the presence or absence of pyrazine. Crucially, therefore, pyrazine odour (or lack of it) accompanied attacks to both prey types: odour never signalled palatability, thus allowing us to separate cueing effects⁶ from other potential psychological responses caused by pyrazine.

In the first experiment, 12 chicks each received 12 sessions of access to 4 green palatable prey and 4 yellow unpalatable prey (sieved chick-starter crumbs spray-dyed with food colourant; yellow crumbs were made unpalatable by soaking in a solution of quinine and mustard) presented individually in sunken wells in a high-walled circular runway (see Methods). Six birds did the experiment in the absence of odour, six in the presence of pyrazine. In the pyrazine group, 2-isobutyl-3-methoxypyrazine solution was added to cotton-wool pads below every well, with holes allowing odour penetration around each prey item. Odour and non-odour groups were tested in duplicate apparatus in duplicate rooms, which were air-filtered and separated from the holding cages. Although both groups of chicks avoided unpalatable prey, discrimination in only the first session was significantly better in the presence of pyrazine (Fig. 1; ANOVA, $F_{1,11} = 19.29$, $P < 0.001$). Thus even though pyrazine was associated equally with both prey types, its presence initially enhanced avoidance of the yellow unpalatable prey.

We considered three explanations. Because pyrazine cannot signal unpalatability in the first experiment, it must either (1) interact with quinine and mustard to enhance the aversiveness of the deterrent, (2) act as a general learning enhancer, or (3) induce a hidden aversion to yellow but not to green prey. In the second experiment, therefore, we repeated the first experiment but added two groups of chicks that experienced the reverse colour discrimination (green unpalatable and yellow palatable). As in the first experiment, the effect of pyrazine is to induce discrimination during the first session. Surprisingly, however, although pyrazine again induces discrimination against yellow unpalatable prey, it also induces discrimination against yellow palatable prey (Fig. 2; ANOVA on the number of correct decisions: colour $F_{1,43} = 18.06$, $P < 0.001$; odour $F_{1,43} = 1.82$, $P < 0.185$; colour $\times$ odour interaction $F_{1,43} = 15.42$, $P < 0.001$). Pyrazine does not, therefore, seem to interact to enhance the aversiveness of quinine and mustard, because discriminative aversion learning with green unpalatable prey was actually retarded in the presence of pyrazine. For the same reason pyrazine cannot be acting as a general

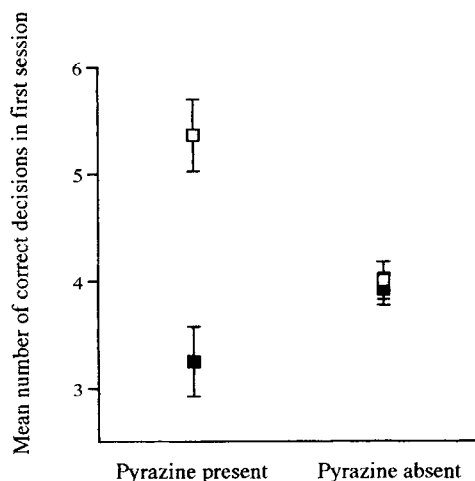


FIG. 2 Mean number of correct decisions ($\pm$ s.e.m.), defined exactly as in Fig. 1, made by each of four treatment groups during the first session of experiment 2 (yellow unpalatable, pyrazine present ($N = 11$); yellow unpalatable, pyrazine absent ($N = 12$); green unpalatable, pyrazine present ($N = 12$); green unpalatable, pyrazine absent ($N = 12$)). Procedures were otherwise as in experiment 1. Open squares: yellow unpalatable and green palatable prey; filled squares: green unpalatable and yellow palatable prey.

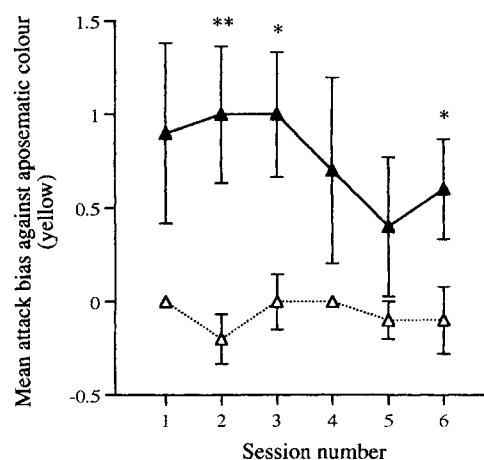


FIG. 3 The results of experiment 4 plotted as the mean attack bias against aposematically coloured crumbs, calculated as yellow crumbs ignored plus green crumbs eaten, in each of six sessions, for groups of chicks with pyrazine present ($N = 10$; filled triangles) or absent ($N = 10$; open triangles). As both crumb colours were palatable, this measure indicates the degree of discrimination against yellow crumbs, with a value of 0 indicating no preference, a value of 4 indicating complete discrimination against yellow, and a value of -4 indicating complete discrimination against green. Procedures were as in experiments 1 and 2. *, $P < 0.05$; **, $P < 0.01$.

learning enhancer, as has been previously hypothesized^{3,10}. Instead, pyrazine odour seems to induce a specific aversion to yellow prey (but not green prey) whichever prey is unpalatable.

We can also conclude from experiment 2 that as quinine and mustard in the absence of odour did not evoke any biased response against yellow, noxiousness alone does not produce the pyrazine effect. In our next two experiments we demonstrated that pyrazine-induced colour aversion does not even require birds to perceive a noxious stimulus in the environment. First we showed that pyrazine is not itself aversive (see also ref. 6). In experiment 3, 13 birds were presented with 6 sessions of access to only familiar, brown, palatable crumbs; 7 birds in the presence of pyrazine and 6 without (other details as experiment 1). There were no differences in the numbers of crumbs eaten between groups either in the first session (ANOVA: $F_{1,12} = 0.85$, $P < 0.377$; pyrazine group, 7.86 ± 0.38 (mean $\pm$ s.e.m.); non-pyrazine group, 8 ± 0 or overall (ANOVA: $F_{1,12} = 1.00$, $P < 0.338$; pyrazine group, $47.57, \pm 0.54$; non-pyrazine group, $46.67, \pm 2.34$). In the next experiment, therefore, we tested the reactions of naive birds in a green versus yellow prey-discrimination task (as in experiments 1 and 2) in the presence or absence of pyrazine, but this time with both prey colours palatable. Pyrazine induces discrimination against yellow prey even in the absence of any noxious stimulus, but yellow and green prey are attacked equally in the absence of pyrazine (Fig. 3; ANOVAs separately on each session showed significantly greater rejection of yellow crumbs in the presence of pyrazine in session 2 ($F_{1,19} = 9.53$, $P < 0.01$), session 3 ($F_{1,19} = 7.50$, $P < 0.05$), and session 6 ($F_{1,19} = 4.74$, $P < 0.05$), and close to significantly greater rejection in session 1 ($F_{1,19} = 3.49$, $P < 0.078$)).

To test the generality of pyrazine's propensity to induce an otherwise hidden aversion to colours characteristic of aposematic prey, we repeated experiment 4 with yellow and green prey but adding two further groups of chicks that received red and green prey. Over the first three sessions of experiment 5, pyrazine significantly enhances aversion to the aposematic colours, as opposed to green, even though all prey are palatable (Fig. 4; ANOVAs separately on each session showed significantly greater rejection of aposematic crumbs in the presence of pyrazine in session 1 ($F_{1,55} = 5.25$, $P < 0.026$), session 2 ($F_{1,55} = 5.96$, $P < 0.018$), and session 3 ($F_{1,55} = 5.60$, $P < 0.021$), and close to

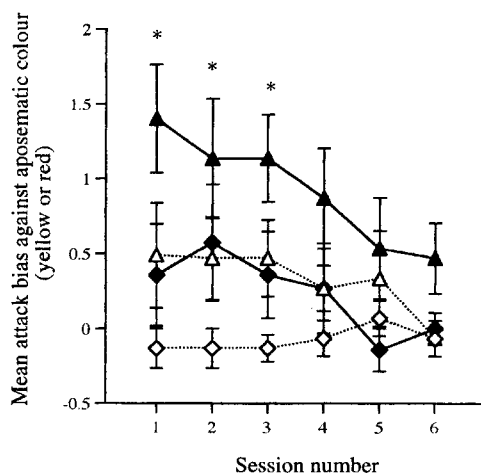


FIG. 4 The results of experiment 5 plotted as the mean attack bias against aposematically coloured crumbs, calculated as yellow (or red) crumbs ignored plus green crumbs eaten, in each of 6 sessions. As all crumbs were palatable, this measure indicates the degree of discrimination against aposematically coloured crumbs (red or yellow), with a value of 0 indicating no preference, a value of 4 indicating complete discrimination against yellow or red, and a value of -4 indicating complete discrimination against green. There were 15 birds in each group except the red with green/pyrazine-present group which had 14. Procedures were as in experiments 1, 2 and 4. Open diamonds: red/green, pyrazine absent; open triangles: yellow/green, pyrazine absent; filled diamonds: red/green, pyrazine present; filled triangles: yellow/green, pyrazine present. Main effect of odour *, $P < 0.05$.

significantly greater rejection in session 4 ($F_{1,55} = 3.64$, $P < 0.062$) and session 6 ($F_{1,55} = 3.72$, $P < 0.059$). Pyrazine has the same effect on red as on yellow prey (there were no significant interactions between odour and aposematic colour type in any session), although a main effect of aposematic colour type indicates that there might be a stronger overall rejection of yellow than of red prey across all four groups (there was a significant main effect of aposematic colour type (red or yellow) in session 1 ($F_{1,55} = 6.99$, $P < 0.011$) and session 3 ($F_{1,55} = 7.93$, $P < 0.007$), and close to a significant effect in session 4 ($F_{1,55} = 3.36$, $P < 0.072$) and session 5 ($F_{1,55} = 3.72$, $P < 0.059$). The impression (from Fig. 4) that bias against aposematic colours wanes during the experiment was tested using Wilcoxon signed ranks tests on the slope distributions of the regression of each chick's performance against session number (H_0 , no slope). Significantly negative slopes were found only for the group encountering yellow and green prey in the presence of pyrazine ($Z = -2.668$; $M = 15$, $P = 0.0076$), suggesting that in nature, unpalatability would ultimately be required to keep the warning signal stable.

Few studies have attempted to analyse the significance of interactions between components in multicomponent signals even though this probably includes most signals¹⁻³. Our experiments demonstrate that pyrazines, which are common components of defensive displays in toxic insects (such as reflex bleeding of coccinellids, or display of *Arctia caja*), trigger in birds unlearned aversions to the typical aposematic colours yellow and red, but not green which is typical of palatable prey. The interaction of odour and colour produces unexpected psychological responses in avian receivers which remain hidden when the components are presented alone, with the clear implication that the full significance of multicomponent animal signals cannot be understood by investigating components independently. ■

Methods

Subjects and stimuli. The subjects were Ross 1 domestic chicks, obtained naive from a commercial hatchery. They were familiarized with the task of

feeding alone in the apparatus, in repeated trials with familiar palatable brown crumbs, for 3 days, before over-night food deprivation and testing on day 4 post-hatching. New birds were used in each experiment. Prey were sieved chick-starter crumbs. Coloration was achieved by spray-dyeing with green, yellow or red food colourant. Prey were either palatable (untainted) or made unpalatable by soaking in a solution of 4% quinine hydrochloride in distilled water, with 2 g mustard powder per 100 ml (then dried). In the pyrazine groups, 3 drops of pyrazine solution (0.1 ml 98% 2-isobutyl-3-methoxypyrazine in 10 ml ethanol and 990 ml distilled water) were added immediately before the start of the experiment to cotton-wool pads under each well in the presentation apparatus. In the non-pyrazine groups, ethanol solution alone was added.

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CORRESPONDENCE and requests for materials should be addressed to T.G. (e-mail: guilford@zoology.oxford.ac.uk).

High incidence of malaria in α -thalassaemic children

T. N. Williams*, K. Maitland*†, S. Bennett‡, M. Ganczakowski*, T. E. A. Peto*, C. I. Newbold*, D. K. Bowden§, D. J. Weatherall* & J. B. Clegg*

* Institute of Molecular Medicine, John Radcliffe Hospital, Headington, Oxford OX3 9DU, UK

‡ Tropical Health Epidemiology Unit, London School of Hygiene and Tropical Medicine, London WC1E 7HT, UK

§ Department of Anatomy, Monash University, Clayton, Victoria 3168, Australia.

THE α^+ -thalassaemias are the commonest known human genetic disorders, affecting up to 80 per cent of some populations¹. Although there is good evidence from both epidemiological^{2,3} and clinical⁴ studies that these gene frequencies reflect selection by, and protection from, malaria, the mechanism is unknown. We have studied the epidemiology of malaria in childhood on the southwestern Pacific island of Espiritu Santo in Vanuatu and here we report that, paradoxically, both the incidence of uncomplicated malaria and the prevalence of splenomegaly, an index of malaria infection, are significantly higher in young children with α^+ -thalassaemia than in normal children. Furthermore, this effect is most marked in the youngest children and for the non-lethal parasite *Plasmodium vivax*. The α^+ -thalassaemias may have been selected for their ability beneficially to increase susceptibility to *P. vivax*, which, by acting as a natural vaccine in this community, induces limited cross-species protection against subsequent severe *P. falciparum* malaria.

The α^+ -thalassaemias are usually caused by the deletion of one of the linked pair of α -globin genes on chromosome 16 (ref. 5). The normal genotype is represented by $\alpha\alpha/\alpha\alpha$. Heterozygotes ($-\alpha/\alpha\alpha$) are clinically normal, whereas homozygotes ($-\alpha/-\alpha$) have a mild anaemia with reduced levels of haemoglobin in red blood cells. To

† Present address: Centre for the Epidemiology of Infectious Disease, Department of Zoology, South Parks Road, Oxford OX1 3PS, UK.

assess the effects of α^+ -thalassaemia on susceptibility to malaria, we studied prospectively the prevalence and incidence of malaria in a cohort of over 900 children living in an area of Santo where the gene frequency of α^+ -thalassaemia is 0.26 and where malaria is hyperendemic⁶. *P. falciparum*, the malignant form of malaria, and *P. vivax*, a relapsing but non-lethal parasite, are the predominant species⁶. We anticipated that any survival advantage afforded by α^+ -thalassaemia might be reflected in a reduced incidence of mild and uncomplicated malaria. We were therefore surprised when the analysis, including all children under 10 years old, demonstrated that the incidence of clinical malaria caused by both of the main species was significantly higher in children homozygous for α^+ -thalassaemia than normal children; the incidence rate ratio (IRR) for clinical *P. vivax* malaria was 2.2 (95% confidence interval, 1.3–2.7; $P = 0.004$) and for clinical *P. falciparum* was 1.6 (1.0–2.6; $P < 0.05$) (Fig. 1a). There was no evidence of an increased incidence in heterozygotes. Further analysis showed that the effect was limited mainly to children less than 5 years old (Fig. 1b and Table 1). In this group the incidence rates for both clinical *P. vivax* and *P. falciparum* malaria in homozygotes were more than twice those in normal children (Fig. 1b). Similar differences in the incidence of fever associated with *P. vivax* or *P. falciparum* parasitaemia of any density were seen between genotypic groups (data not shown), showing that these observations were not biased by the criteria used to define clinical malaria. The conclusion that these children are predisposed to malaria is supported by indirect evidence from rates of splenomegaly determined from cross-sectional surveys (Table 2). Both homozygous ($-\alpha/-\alpha$) and heterozygous ($-\alpha/\alpha\alpha$) children were significantly more likely to have palpable spleens than were normal children; the relative risk of splenomegaly was 1.5 in homozygotes and 1.2 in heterozygotes (Table 2). Furthermore, when examined by forward stepwise logistic regression analysis, the data show that although splenomegaly was associated with the presence of malaria parasites, it was not independently associated with α^+ -thalassaemia, indicating that the increased rate of splenomegaly among these children resulted from a raised incidence of malaria. Parasite prevalence data collected concurrently by cross-sectional survey did not demonstrate a consistent pattern (Table 2).

The only other clinical study to address the relationship between homozygous α^+ -thalassaemia and malaria offers some support for our observations. During a controlled trial of iron dextran prophylaxis in infants from Papua New Guinea, raised spleen and parasite rates were found in cross-sectional surveys of homozygous children at 6 months and 12 months of age⁷. It was suggested that this was caused by a slower rate of parasite growth in thalassaemic red blood cells, resulting in a greater persistence of infection, a theory apparently disproved by subsequent *in vitro* studies⁸.

These findings are difficult to reconcile with the concept of selection for α -thalassaemia by malaria. The most obvious conclusion is that the hypothesis is wrong; however, not only is the epidemiological evidence for protection extremely strong^{2,3}, but in a case-control study we have recently conducted in Papua New Guinea (another Melanesian population), we found that homozygous α^+ -thalassaemia conferred significant protection against severe malaria in childhood (S. J. Allen *et al.*, manuscript in preparation). That study gives no insight into the mechanism involved whereas the Vanuatu findings suggest that it may have an immunological basis. Like other mild forms of thalassaemia, α^+ -thalassaemia is probably associated with some degree of ineffective erythropoiesis and reduced red blood cell survival⁹, resulting in a higher proportion of circulating young red blood cells. As a consequence, it is likely that infection with both *P. falciparum*, which has a preference for the youngest, most metabolically active red cells¹⁰, and *P. vivax*, which only invades reticulocytes, would be more efficient. We speculate that this may be one way in which α^+ -thalassaemia results in an increased incidence of malaria in childhood. During early infancy severe malaria is rare, probably because children are partially protected by passive immunity from

maternal antibodies and raised levels of fetal haemoglobin¹¹. An increased susceptibility to malaria during this period may result in improved immunity to subsequent severe disease. The observations of ref. 12 are important in this respect: *in vitro*, surface antigen expression in *P. falciparum*-infected α -thalassaemic red blood cells is almost twice that of infected normal red cells, a phenomenon that may lead to more efficient presentation of parasite antigens to the immune system. We have limited evidence of improved immunity in homozygotes, from the reduced (although not statistically significant) incidence of clinical *P. falciparum* infection seen in homozygotes over 5 years old (Fig. 1b).

Two further observations are relevant. First, the incidence of *P. vivax* malaria peaks earlier in life in this population than that of *P. falciparum*⁶, a recurrent finding in communities where the two parasites co-exist^{13–15}. Second, the raised incidence of *P. vivax* malaria seen in homozygotes is most marked in the youngest children (IRR = 3.9 in homozygotes under 30 months; 95% confidence interval 1.9–8.1; $P = 0.0001$). There is some evidence that infection with *P. vivax* may lead to limited immunity to subsequent infection with *P. falciparum*^{16,17}. Such immunity, if it occurs, is clearly not complete: children in Vanuatu suffer *P. falciparum* malaria despite previous attacks of *P. vivax*; however, *P. vivax* may induce a degree of cross-species immunity sufficient to reduce the risk of subsequent severe *P. falciparum* infection. Such a process could be enhanced in infants homozygous for α^+ -thalassaemia as a consequence of their increased susceptibility to *P. vivax*.

Population genetic data suggest that the predominant fitness advantage in α^+ -thalassaemia is limited to homozygotes and that the fitness increment associated with heterozygosity may be less

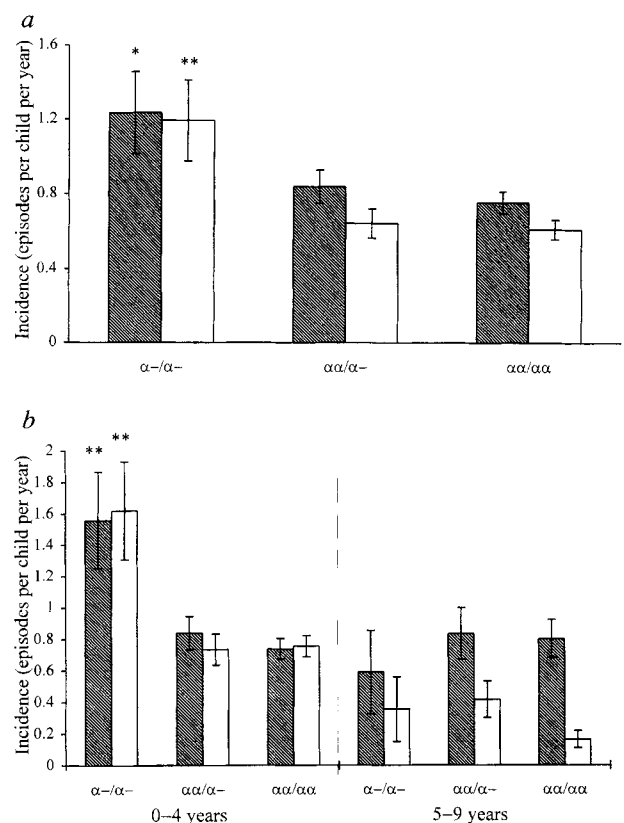


FIG. 1 The incidence of clinical malaria by α -thalassaemia genotype. a, Crude annual incidence rates (with standard errors), by genotype, for *P. falciparum* (shaded bars) and *P. vivax* (white bars) malaria (all ages) and b, split by age group. Significance levels were derived using negative binomial regression (Table 1). * $P < 0.05$, ** $P < 0.005$.

TABLE 1 Incidence rate ratios for clinical malaria by genotypic group and age groups

	<5 years old			≥ 5 years old		
	−α/−α	−α/αα	αα/αα	−α/−α	−α/αα	αα/αα
Number of children	36	171	337	34	115	214
Number of observations	867	3,961	8,729	440	1,660	2,893
Episodes of clinical <i>P. falciparum</i>	26	64	124	5	26	44
IRR for <i>P. falciparum</i>	2.3 (1.32–4.07) P = 0.003	1.1 (0.77–1.61) P = 0.56	1.0	0.7 (0.27–1.90) P = 0.50	1.1 (0.62–1.90) P = 0.77	1.0
Episodes of clinical <i>P. vivax</i>	27	56	127	3	13	9
IRR for <i>P. vivax</i>	2.4 (1.40–4.30) P = 0.002	1.0 (0.66–1.40) P = 0.83	1.0	2.0 (0.42–9.14) P = 0.39	2.5 (0.94–6.82) P = 0.07	1.0

Incidence rate ratios (IRR) for clinical *P. falciparum* and *P. vivax* malaria by age and genotypic group adjusted for confounding variables. 95% confidence intervals (in parentheses) and significance levels (P) were calculated by negative binomial regression.

than 0.02 (ref. 18). We have found no effect of heterozygosity on malaria incidence, an observation which supports this hypothesis. We and others¹⁹ have shown, however, that heterozygotes have a higher prevalence of parasitaemia on cross-sectional survey. In addition, we have demonstrated a raised prevalence of splenomegaly in this group. It may be that these are the only detectable manifestations of a raised incidence of disease and that the incremental advantage conferred upon heterozygotes is too small to be detected in a study of this size. Accepting that malaria is responsible for selecting α^+ -thalassaemia in this population, either the current gene frequency of 0.26 will, given sustained malaria pressure, continue to increase towards fixation, or an equilibrium has already been reached owing to a (presently unknown) balancing disadvantage of the condition.

Our findings raise some important issues. First, while highlighting how little is understood about the development of protective immunity to *P. falciparum*, they imply that this process is improved by higher rates of infection in early life. This possibility has obvious implications for interventions that result in reduced exposure to malaria in childhood and emphasize the need for very careful long-term monitoring of such programmes. Second, they offer indirect evidence of cross-species protection. *P. vivax* may be

acting as a natural vaccine in areas where the two parasites co-exist. Because this parasite remains exquisitely sensitive to most antimalarials it is possible that misuse of these drugs could result in increased severe malaria caused by *P. falciparum*.

Finally, it is noteworthy that infectious diseases such as rubella and poliomyelitis cause least morbidity when contracted early in childhood. It is possible that α^+ -thalassaemia provides the first example of a genetic condition selected for its ability beneficially to increase susceptibility to an infectious agent. □

Methods

Study area. The study was conducted on the island of Santo, Vanuatu in the southwest Pacific. Malaria transmission is perennial but more than 80% of new cases caused by *P. falciparum* occur during the 'wet season' (November–May) whereas more than 65% of cases of *P. vivax* occur in the 'dry season' (June–October). *P. falciparum* malaria seems to follow a relatively mild course in this community, with a very low incidence of severe and complicated disease and a low fatality rate⁶.

Study population. The study was conducted between January 1992 and November 1993. All children under 10 years old, from consenting families, were recruited from a total of 10 villages hyperendemic for malaria. Recruitment of newborns and migrants continued throughout the study; however, children over 5 years old were not recruited until the second year of the study (November 1992–November 1993). The α^+ -thalassaemia gene frequency is 0.26 (ref. 20). Full accounts of the population distribution of these conditions, their clinical effects and underlying molecular pathology have been reported previously^{2,20,21}. The prevalence of other conditions thought to confer protection against malaria^{22–25} is low; sickle-cell anaemia²², Melanesian ovalocytosis²³ and β -thalassaemia are absent and the gene frequency of glucose-6-phosphate dehydrogenase deficiency is only 0.03 in males. Venous blood was collected from all children for later determination of α -thalassaemia status by the Southern blot procedure²⁶. The children's genotypes were unknown to the investigators throughout the period of data collection.

Surveillance. Cohort children were followed weekly by trained village-based field workers. At follow-up they completed a standard morbidity questionnaire detailing the child's state of health, any clinical symptoms and recent medications and recorded axillary temperatures. Thick and thin blood films for malaria parasites were prepared on children with either a history of fever in the preceding 48 h or an axillary temperature over 37.4 °C. Suspected cases of malaria were then treated prospectively.

Cross-sectional surveys. Throughout the study period, random samples of cohort children were screened periodically for the prevalence of splenomegaly and malaria parasitaemia. Splenomegaly was assessed by a single observer by standard methods and graded using Hackett's system²⁷. The only common cause of splenomegaly in this area is malaria. In particular, α^+ -thalassaemia is not known to be associated with an enlarged spleen in the absence of malaria. Parasite densities were measured on Giemsa-stained thick blood films by standard methods.

Statistical analysis. The main outcomes of interest were the incidence rates of clinical *P. falciparum* and *P. vivax* malaria. Previous work has determined case definitions for clinical malaria by multiple logistic regression⁶. Clinical episodes were defined as fever (either a history of fever in the preceding 48 h or a measured axillary temperature over 37.4 °C) in the presence of asexual parasitaemia over 1,000 μl^{-1} for *P. falciparum* and over 500 μl^{-1} for *P. vivax* malaria. The unadjusted annual incidence rates for malaria were calculated using the formula: incidence = (total number of episodes/total number of observations) $\times$ 52 weeks. The length of follow up was 85 weeks for children 0–4 years and 48 weeks for children 5–9 years. Children were considered not at risk and dropped from numerator and denominator populations for 28 d after treatment with

TABLE 2 The prevalence of splenomegaly and parasitaemia in children of different α -thalassaemia genotype

	−α/−α	−α/αα	αα/αα
Splenomegaly			
n	48	145	358
Spleen palpable (%)	65	52	43
RR	1.5 (1.2–1.9) P = 0.004	1.2 (1.0–1.5) P = 0.05	1.0
Parasitaemia			
Wet season			
n	60	253	485
<i>P. falciparum</i> (%)	28	37	30
RR	1.0 (0.6–1.5) P = 0.8	1.2 (1.0–1.6) P = 0.04	1.0
<i>P. vivax</i> (%)	5	16	12
RR	0.4 (0.1–1.3) P = 0.1	1.35 (0.9–2.0) P = 0.1	1.0
Dry season			
n	31	94	254
<i>P. falciparum</i> (%)	10	6	7
RR	1.5 (0.5–4.7) P = 0.5	1.0 (0.4–2.4) P = 0.9	1.0
<i>P. vivax</i> (%)	26	20	20
RR	1.3 (0.7–2.5) P = 0.4	1.0 (0.6–1.6) P = 0.9	1.0

The prevalence and relative risk (RR) of splenomegaly and malaria parasitaemia by α -thalassaemia genotype for children 0–10 years (95% confidence interval in parentheses). n, number of individuals screened, only the first assessment being included for individuals screened more than once.

antimalarials for confirmed or suspected malaria. However, if any of the latter children became a confirmed case during this exclusion period, it was assumed that treatment had failed and this exclusion period was discounted. Incidence rates of clinical malaria for $-x/-x$ and $-x/xx$ were compared separately to those for children of normal genotype and are presented as incidence rate ratios (IRR) adjusted for confounding variables (village of residence and sex) (Table 1). Confidence intervals and significance levels were calculated using a negative binomial regression model to account for within-patient clustering of episodes. This model inflates standard errors and confidence intervals to allow for inter-person variability. Preliminary analysis with a Poisson regression model, which assumes independence of events, did not materially alter the results. The analyses presented were conducted separately for children under 5 years and 5 years or over. The differences seen in the two age groups were not a consequence of the disparity in the duration of follow-up of these groups. A separate analysis of incidence for the period November 1992–November 1993 (not shown), during which children of all ages were followed, showed similar results. The analysis was performed using STATA V 4.0 for Windows (Timberlake, UK). To investigate which variables were independently associated with spleen enlargement a forward stepwise logistic regression model was fitted for the categorical variables: age group (less than or greater than 5 years); sex; the presence of asexual forms of *P. falciparum*; the presence of asexual forms of *P. vivax*; fever and α -thalassaemia genotype. Although the presence of malaria parasites and fever were associated with spleen enlargement, thalassaemia genotype was not independently associated. Differences in parasite and splenomegaly rates were compared between thalassaemic groups and normals by χ^2 analysis.

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CORRESPONDENCE and requests for materials should be addressed to T.N.W. (e-mail: tom.williams@imm.ox.ac.uk).

Semaphorin III is needed for normal patterning and growth of nerves, bones and heart

Oded Behar*, Jeffrey A. Golden†, Hiroshi Mashimo*, Frederick J. Schoen† & Mark C. Fishman*

* Cardiovascular Research Center, Massachusetts General Hospital (East), 149 13th Street, 4th Floor, Charlestown, Massachusetts 02129, and Department of Medicine, Harvard Medical School, 25 Shattuck Street, Boston, Massachusetts 02115, USA

† Department of Pathology, Brigham and Women's Hospital, Harvard Medical School, 75 Francis Street, Boston, Massachusetts 02115, USA

THE expression patterns of the recently discovered family of semaphorin genes suggests that they have widespread roles in embryonic development^{1–9}. Some seem to guide neuronal growth cones, but otherwise their functions are unknown^{2,10–12}. Semaphorin III is a membrane-associated secreted protein with a developmentally dynamic pattern of expression, including particular domains of the nervous system, the borders of developing bones, and the heart^{6,7}. *In vitro*, semaphorin III causes growth-cone collapse, and repels cutaneous sensory axons from the ventral spinal cord^{2,11}. Mutants in the *Drosophila* gene *semaII*, which encodes a related semaphorin, die after eclosion, but no responsible abnormality is evident³. We have generated mice mutant in the *semaIII* gene by homologous recombination. Here we show that in the mutants, some sensory axons project into inappropriate regions of the spinal cord where *semaIII* is normally expressed. The cerebral cortex of homozygous mutant mice shows a paucity of neuropil and abnormally oriented neuronal processes, especially of the large pyramidal neurons. Certain embryonic bones and cartilaginous structures develop abnormally, with vertebral fusions and partial rib duplications. The few mice that survive more than a few days postnatally manifest pronounced and selective hypertrophy of the right ventricle of the heart and dilation of the right atrium. Thus, semaphorin III might serve as a signal that restrains growth in several developing organs.

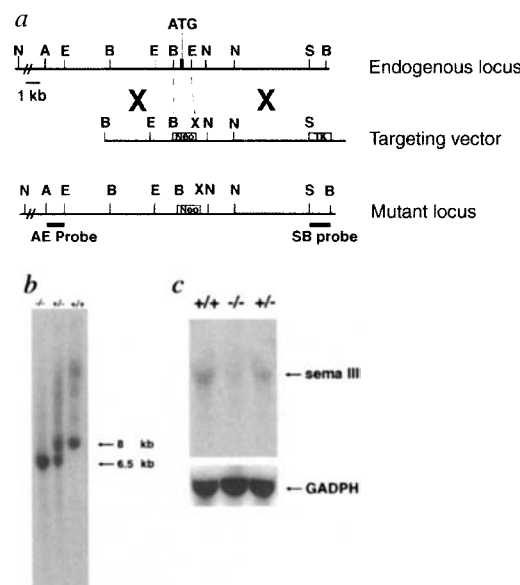
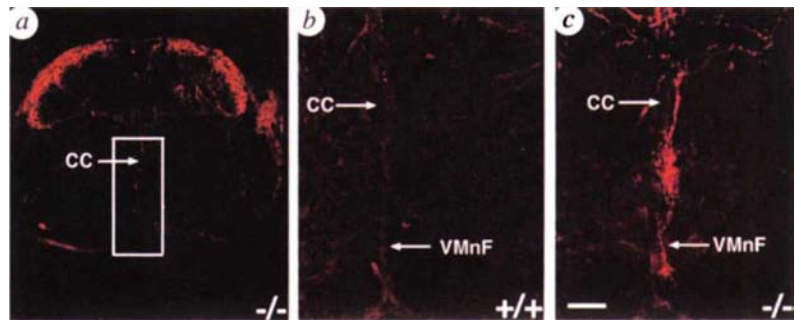


FIG. 1 Targeted disruption of the *semaIII* gene. *a*, Restriction map of the native mouse *semaIII* gene, the targeting vector, and the disrupted *semaIII* gene. The targeting vector contains 5' and 3' flanking regions of homology, and is designed to replace the *Bam*HI to *Eco*RI fragment which contains the first coding exon (amino acids 1–38). E, *Eco*RI; B, *Bam*HI; S, *Sma*I; A, *Sau*3A1; N, *Nco*I and *Bam*HI. *b*, Southern blot analysis of genomic DNA, digested with *Bam*HI and hybridized with the external 3'-flanking SB probe, which is external to the targeting vector. The SB probe recognizes a 8-kb hybridizing fragment in the native gene and a 6.5-kb hybridizing fragment in the disrupted gene. Proper insertion was demonstrated as well by digestion with *Nco*I and hybridization with the 5'-flanking AE probe (data not shown). The positions of the hybridizing fragments for the wild-type (8 kb) and the disrupted alleles (6.5 kb) are shown. *c*, Northern blot analysis using mouse *semaIII* complementary DNA fragment (bases 1 to 623) as a probe. The same RNA blot was rehybridized with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA to verify equivalent RNA loading.

FIG. 2 Abnormal organization of the spinal cord in mutant mice. *a–c*, CGRP (for calcitonin-gene-related peptide) immunohistochemistry of newborn lumbar spinal cord of (*a*, *c*) homozygous mutant, and (*b*) wild-type mice, examined with confocal microscopy. *b* and *c* are enlargements of the boxed area in *a*. Dorsal spinal cord is up. Arrows mark the central canal (CC) and the ventral median fissure of the spinal cord (VMnF). Note the abnormal ventral extension of CGRP-immunoreactive fibres. (This extension was noted in 9 of 10 homozygous mutant mice and none of 9 heterozygous and wild-type mice). Scale bars: *a*, 187 μ m; *b*, *c*, 52 μ m.



We generated mice mutant in *semaphorin III/collapsin1* using embryonic stem cells in which we replaced the first coding exon with the *neo* cassette. The deleted exon includes 9 bases of 5'-untranslated region and those encoding the amino-terminal 38 amino acids, including the presumptive signal sequence of semaphorin III (SemaIII) (Fig. 1*a*). Correct homologous insertion was confirmed by Southern blot analysis, using both 5'- and 3'-flanking probes (Fig. 1*b*, and data not shown), and deletion of the targeted exon confirmed by polymerase chain reaction (PCR) in all homozygous mice (data not shown). The amount of *semaIII* RNA detected by northern blot analysis is about half that of the wild type in heterozygous mice and is barely detectable in homozygous mice (Fig. 1*c*). The RNA size of 5 kilobases is indistinguishable from wild type, as predicted by the deletion of 121 base pairs, and no new hybridizing bands are noted.

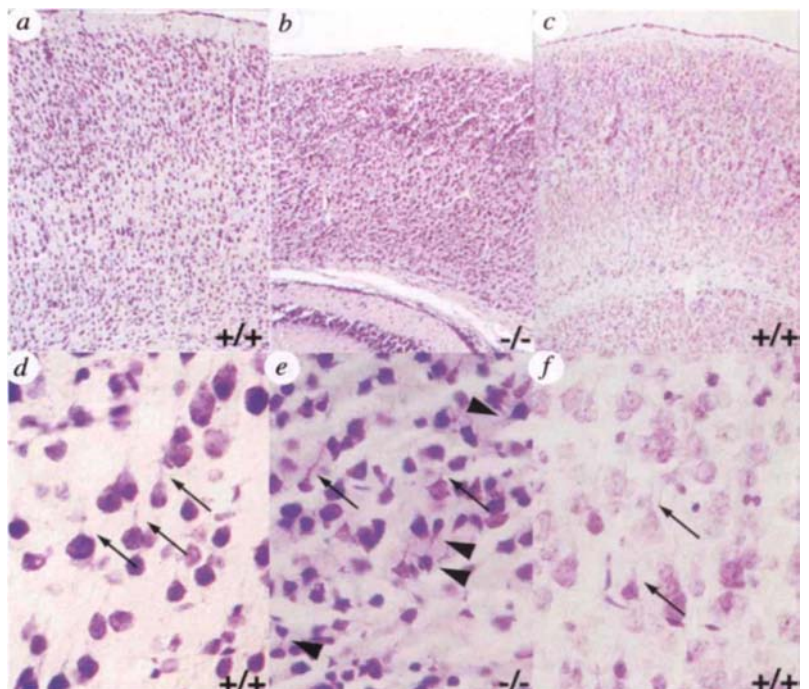
Heterozygous mice appear normal. Homozygous deficient mice are born at slightly lower than predicted mendelian ratios (19% homozygous, 31% wild type, 46% heterozygous, 4% not scored; $n = 378$). At birth, homozygous progeny are indistinguishable from their littermates. However, within 24 hours many of the homozygous mice seem weaker and have less milk in their stomachs, and most (70%) die within the first 3 days. Another 17% die at weaning and about 1% die over the following 3 months. A small percentage of the homozygous progeny (12%) are viable to adulthood. The surviving homozygous mice are smaller than wild type and have difficulty maintaining upright posture.

SemaIII is believed to repel nerve growth factor (NGF)-depen-

dent thermoceptive and nociceptive sensory axons from inappropriate targets in the ventral spinal cord^{11,13}. During the period of gestation in the rat and chick when these dorsal root ganglion (DRG) axons enter the cord and reach their laminar targets, *semaIII* is expressed in the ventral spinal cord, especially in the periventricular zone and in motor columns^{6,7,11}. Many of these NGF-dependent nerves are calcitonin-gene-related peptide (CGRP)-immunoreactive, and their axon terminals are concentrated in dorsal laminae, as shown in Fig. 2*a*¹⁴. In *semaIII* homozygous mutant mice, CGRP-immunoreactive axons often extend outside their normal terminal zones (Fig. 2*b*, *c*), reaching the central canal and medial ventral cord. The relatively normal histology of the spinal cord and neurofilament immunostaining (data not shown) indicates that the overall pattern of tracts is retained, and that there are guidance mechanisms in addition to SemaIII.

Messenger RNA from the *semaIII* gene is normally present at high levels in the developing brain^{6,7}. The brains of postnatal day 12 (P12) homozygous deficient mice are smaller than their wild-type littermates, and comparable to P7 wild-type mice. Although all regions of the brain are reduced in size, histological examination reveals no obvious abnormalities in the deep grey nuclei, cerebellum, or brainstem. The thickness of the cerebral cortex is roughly 70% that of the wild type and comparable to the thickness of the P7 wild type cortex (Fig. 3*a–c*). Although less clearly demarcated than wild type, all cortical layers are present. The neuropil, composed of axons, dendrites and glial processes

FIG. 3 Abnormal organization of the central nervous system in mutant mice. *a–f*, The cerebral cortex of postnatal (*a*, *d*) day 12 $+/+$, (*b*, *e*) day 12 $-/-$ and (*c*, *f*) day 7 $+/+$ mice stained with the Nissl method. All sections are oriented such that the pial surface is up and the white matter is down. Arrows in *d*, *e* and *f* show the normal orientation of processes for pyramidal cells. Large arrowheads show misoriented processes in the $-/-$ mice. Magnifications in *a*, *b* and *c* are $\times 100$, and in *d*, *e* and *f* are $\times 400$.



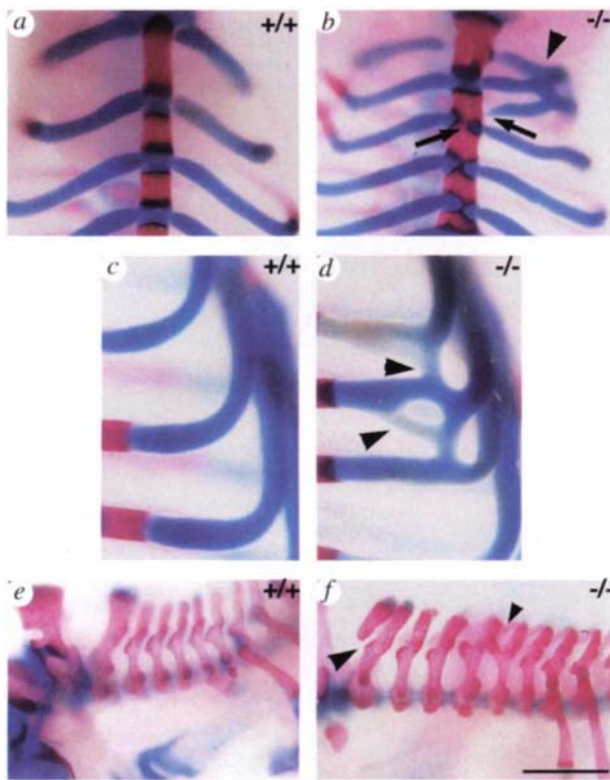


FIG. 4 Bone and cartilage abnormalities in *semalIII* homozygous mutant mice. *a*, Sternum of wild type, showing orderly insertion pattern of ribs, in contrast to *b*, homozygous mutant mice, which evidence misalignment of the rib-sternal junction and an irregular border to the sternum itself. Arrowhead in *b* indicates area of rib fusion and duplication. Arrows indicate misalignment of the ribs with the sternum. Thoracic region of (*c*) wild-type and (*d*) mutant mice, showing rib fusion and partial duplication. Cervical region of (*e*) wild-type and (*f*) homozygous mice, showing vertebral fusion and split. Arrowheads indicate the point of fusion. Scale bars: *a*, *b*, 1.74 mm; *c*, *d*, 1 mm; *e*, *f*, 1.6 mm.

located between neurons, is severely reduced (Fig. 3*b, e*), and neuronal morphology is altered in the homozygous deficient mice. The pyramidal neurons, especially the large pyramidal neurons of layer 5, are normally oriented with a dendritic helix pointing towards the surface of the brain (Fig. 3*d, f*). In the mutant mice, many neurons are found with processes pointing in random directions, in addition to those neurons with normally directed processes (Fig. 3*e*). Misoriented neuronal processes of similar features are found in human disorders such as cortical dysplasia and type 1 lissencephaly¹⁵.

In the embryonic rat, *semalIII* mRNA is expressed in the sclerotome, which gives rise to chondrocytes of the axial skeleton⁶. Later in development, *semalIII* is expressed in the developing ribs and pelvic girdle. The gene is down-regulated in differentiated bone, but the level of expression remains high in the bone margins, including those of the ribs and vertebral column⁶. *semalIII* homozygous mutant mice display fusion of cervical bones (Fig. 4*e, f*), partial duplications of ribs (Fig. 4*a-d*), and poor alignment of the rib-sternum junction (Fig. 4*a, b*). The sternum is thickened and irregular and the xiphoid may be split. The *semalIII* gene is expressed in dermamyotome and subsequently around muscles, limb and dermis⁶ but, using standard histological techniques, we do not find abnormalities of these tissues.

There is expression of *semalIII* mRNA in the developing rat heart⁶. The rare homozygous mutant mice that survived the first days of life tended to die during the next 3 weeks, and all have evidence of fulminant right-heart failure ($n = 14$). The right atrium is grossly dilated, and generally filled with clot (Fig. 5*a*,

b). The right ventricle is enlarged and its wall, normally thin by this age, is hypertrophied, such that it is equivalent to the left in thickness (Fig. 5*c, d*). (The right and left ventricles are more similar in thickness at birth, both in wild-type and in ten mutant animals that died in the first 3 days after birth.) There is no evidence of obstruction of the right ventricular outflow tract or pulmonic valve. No pulmonary emboli or pulmonary artery hypertrophy is found, suggesting that there is no long-standing pulmonary hypertension, although intermittent physiological increments would not be evident.

The pronounced phenotype of these mice shows that *SemaIII* has important developmental roles in several tissues, all of which normally express *semalIII*. We presume that these effects reflect absence of protein, given the fact that the deleted region includes the presumptive translational start and secretory signal sequence, and the observation that RNA levels are barely detectable by northern blot. However, we cannot exclude the formal possibility of truncated protein synthesis, for example from a cryptic translational start site¹⁶. Collapsin 1, the chick homologue of *SemaIII*, causes growth-cone collapse, and human *SemaIII* repels NGF-dependent sensory neurons^{2,11}. The invasion of anomalous territories by NGF-dependent DRG neurons is compatible with a normal role for *SemaIII* in demarcating boundaries to growth of particular axons. *SemaIII* also seems to sculpt borders of bones, compatible with its early embryonic expression in sclerotome, and later at bone margins. *BMP-7* mutant mice manifest similar bony abnormalities in the ribs and sternum¹⁷, but whether the two factors affect similar pathways has not been investigated.

Many of the *SemaIII*-deficient mice die with right heart failure. The normal right ventricle differs from the left ventricle in growth properties and structure. There are different patterns of gene expression between the left and right ventricles from very early embryonic stages¹⁸. After birth, the right ventricle supports the pulmonary circulation, and therefore is required to generate much lower pressures than is the left ventricle. If there is pulmonary

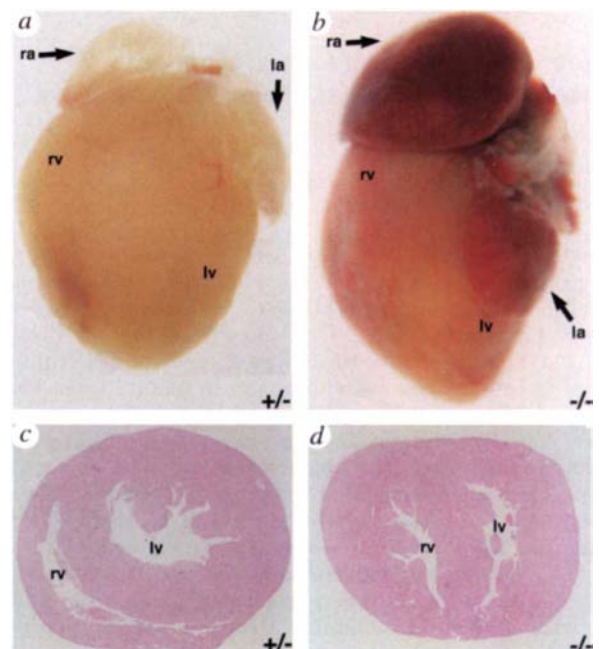


FIG. 5 Right-sided cardiac hypertrophy in homozygous deficient mice. *a*, Wild-type, postnatal day 10. *b*, Homozygous mutant, postnatal day 10, showing massive dilatation of the right atrium, which is filled with clot, and enlargement of the right ventricle. *c*, Wild-type, cross-section, stained with haematoxylin and eosin showing that the normal right ventricular wall is much thinner than the left. *d*, Homozygous mutant, showing right ventricular hypertrophy.

hypertension, the right ventricles dilates and, over time, hypertrophies and may fail. However, we have no evidence that the right ventricular hypertrophy is secondary to pulmonary hypertension, in that there is no embolic pulmonary vascular obstruction, diffuse lung disease, or congenital cardiac malformation, and the outflow tract and valves appear normal. One interpretation is that normally SemaIII represses growth of the right ventricle. It is also plausible that absence of SemaIII directly or indirectly enhances the right ventricular growth response to even minor overload, as might occur, for example, if the rib abnormalities cause hypoxaemia by limiting normal thoracic ventilatory excursion. Little is known about growth controls of the ventricular myocyte or of the transition from hypertrophy to failure. This mutant suggests that semaphorin III may be one cardiac-growth-regulating signal, and the mutant may provide a system in which it can be evaluated. □

Methods

Generation of mutant mice. The J1 embryonic stem cell line was derived and grown as described¹⁹. Electroporated cells were selected in G418 and FIAU (1-[2'-deoxy-2'-fluoro- β -D-arabinofuranosyl]-5-iodouracil)²⁰, the latter to select against retention of thymidine kinase (TK) and hence for homologous recombination. Resistant colonies were analysed by Southern blot²⁰. The 5' probe was the *Sau3AI*-*EcoRI* (AE) fragment and the 3' probe was the *SpeI*-*BamHI* (SB) fragment (shown in Fig. 1a). Total RNA (20 μ g) from P1 brain of wild-type heterozygous and homozygous mutant mice was electrophoresed, blotted and hybridized as described²¹.

Histology. Postnatal day 1, 7 and 12 *SemaIII* $-/-$ and $+/+$ animals were perfused with 4% paraformaldehyde. Spinal cords and brains were dissected and postfixed for 12 h. For immunostaining, lumbar spinal cords were equilibrated with 20% sucrose, sectioned at 20 μ m and stained with rabbit anti-CGRP

antibody diluted 1:500 (Amersham), followed by incubation with FITC-conjugated goat anti-rabbit antiserum (1:100). The sections were examined using confocal microscopy. Alizarin red and alcian blue staining were used for skeletal analysis²². For Nissl staining, brains were embedded in paraffin and serially sectioned in the coronal plane. Every tenth slide was stained with the Nissl method and examined by light microscopy.

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CORRESPONDENCE and requests for materials should be addressed to M.C.F. (e-mail: fishman@cvcrc.mgh.harvard.edu).

c-Cbl is downstream of c-Src in a signalling pathway necessary for bone resorption

Sakae Tanaka, Michael Amling, Lynn Neff, Anusch Peyman*, Eugen Uhlmann*, Joan B. Levy & Roland Baron

Departments of Cell Biology and Orthopedics, Yale University School of Medicine, New Haven, Connecticut 06510, USA

* Hoechst AG, Central Pharma Research, 65926 Frankfurt, Germany

THE primary defect in mice lacking the *c-src* gene is osteopetrosis, a deficiency in bone resorption by osteoclasts¹. Osteoclasts express high levels of the *c-Src* protein^{2,3} and the defect responsible for the osteopetrotic phenotype of the *c-src*-deficient (*src*⁻) mouse is cell-autonomous and occurs in mature osteoclasts^{4,5}. However, the specific signalling pathways that require *c-Src* expression for normal osteoclast activity have not been elucidated. We report here that the proto-oncogene product *c-Cbl* is tyrosine-phosphorylated in a *Src*-dependent manner in osteoclasts, where the two proteins colocalize on some vesicular structures. *In vitro* bone resorption by osteoclast-like cells (OCLs) is inhibited by both *c-src* and *c-cbl* antisense oligonucleotides. Furthermore, tyrosine phosphorylation of *c-Cbl* and the localization of *c-Cbl*-containing structures to the peripheral cytoskeleton are impaired in resorption-deficient *c-src*⁻ OCLs, as well as in wild-type OCLs that have been treated with *c-src* antisense oligonucleotides. These results indicate that *c-Cbl* may act downstream of *c-Src* in a signalling pathway that is required for bone resorption.

The product of the proto-oncogene *c-Cbl* is a protein of relative molecular mass 120K that is tyrosine-phosphorylated in response to the activation of various signalling pathways and in *v-Src*

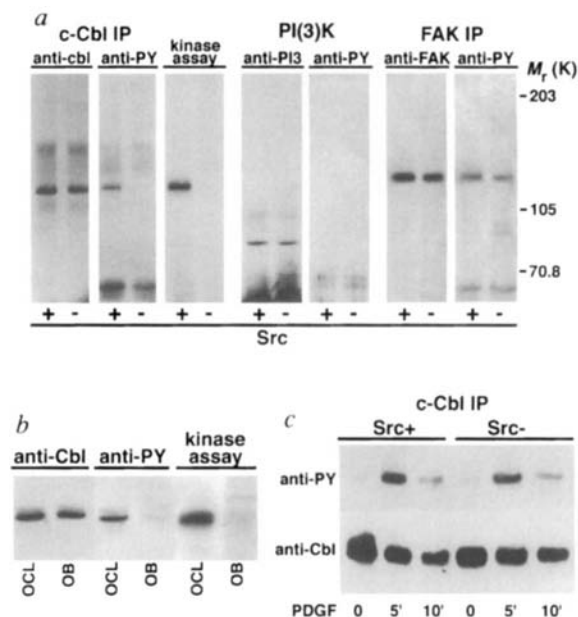


FIG. 1 *c-Cbl* requires *c-Src* to be phosphorylated in osteoclast-like cells. **a**, *c-Cbl*, immunoprecipitated (*c-Cbl* IP) from *Src*⁺ and *Src*⁻ OCLs, was immunoblotted with anti-*c-Cbl* antibody (anti-cbl) and anti-phosphotyrosine antibody (anti-PY). The immune complexes were also analysed by *in vitro* kinase assay. Although under basal conditions the level of *c-Cbl* tyrosine phosphorylation was reduced in *Src*⁻ OCLs, other known substrates of *c-Src* were normally tyrosine-phosphorylated, as shown here for p85 PI(3)K (PI(3)K IP) and p125 FAK (FAK IP); **b**, *c-Cbl* immunoprecipitates from osteoblasts (OB) show that in OBs *c-Cbl* is not phosphorylated or associated with a kinase; **c**, Lung fibroblasts obtained from *c-Src*-deficient mice (*Src*⁻) or their normal littermates (*Src*⁺) respond similarly to 2nM PDGF, with rapid phosphorylation of *c-Cbl*.

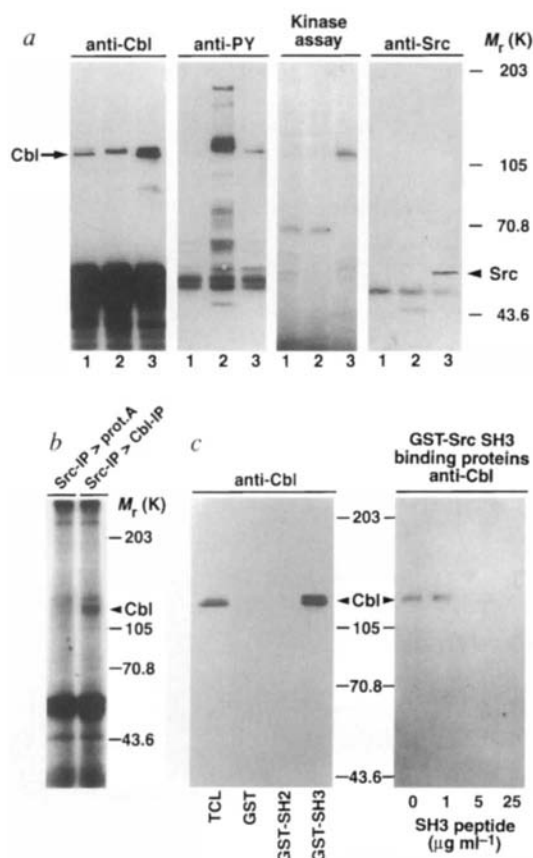


FIG. 2 Interactions of c-Cbl and c-Src in OCLs. *a*, c-Cbl immunoprecipitates from c-Src-overexpressing fibroblasts (lane 1), v-Src-transformed fibroblasts (lane 2) and OCLs (lane 3) were immunoblotted with anti-c-Cbl antibody (anti-Cbl), anti-phosphotyrosine antibody (anti-PY) and anti-Src antibody (anti-Src), as well as tested in the *in vitro* kinase assay. c-Cbl (arrow) and c-Src (arrowhead) are indicated. c-Src is present in c-Cbl immunoprecipitates of OCLs but not of other cells. *b*, c-Cbl is immunoprecipitated from the Src immune complex after kinase assay, confirming the association of c-Src/c-Cbl in OCLs. *c*, Left, immunoblotting of total-cell lysates (TCL) and GST, GST-Src SH2, and GST-Src SH3-binding proteins in OCLs with anti-c-Cbl antibody; right, competition analysis of the binding of c-Cbl to the Src-SH3 domain with a peptide that specifically binds to Src-SH3.

transformed cells⁶⁻¹³. To determine the role of c-Src in this phosphorylation and the functional role of this pathway in bone resorption, we did a comparative analysis of c-Cbl phosphorylation in Src⁺ and Src⁻ OCLs, osteoblasts and fibroblasts, and found that c-Cbl is tyrosine-phosphorylated in a Src-dependent manner, and that this dependence may be specific to OCLs. This conclusion is based on the facts that (1) the level of tyrosine phosphorylation of c-Cbl immunoprecipitated from Src⁻ OCLs was strikingly reduced when compared with that found in wild-type OCLs (Fig. 1*a*; anti-PY), even though the amount of c-Cbl protein was the same in Src⁺ and Src⁻ OCLs (Fig. 1*a*; anti-Cbl). This reduced level of tyrosine phosphorylation was not observed for known substrates of c-Src: under basal culture conditions, when integrin receptors are engaged through cell attachment, p85 phosphatidylinositol-3-OH kinase (PI(3)K; Fig. 1*a*), cortactin, p120 and p130Cas (not shown) are not phosphorylated in OCLs, whereas p125 FAK (ref. 14) is tyrosine-phosphorylated in both Src⁺ and Src⁻ OCLs (Fig. 1*a*). Thus, c-Cbl but not all Src substrates show a defective phosphorylation pattern in OCLs in the absence of c-Src. (2) c-Cbl immunoprecipitated from Src⁻ OCLs was not phosphorylated in an *in vitro* kinase assay, when c-Cbl immunoprecipitated from Src⁺ cells could be further phosphorylated *in vitro* upon addition of [γ -³²P]ATP (Fig. 1*a*; kinase assay), predominantly on tyrosine residues (data not shown)¹⁵. This implies that, after genetic deletion of c-src, no tyrosine-kinase activity associates with c-Cbl in OCLs, whether this kinase is c-Src itself or another kinase whose association with c-Cbl or activity is c-Src-dependent.

We then attempted to determine whether c-Cbl directly associates with c-Src. *In vivo*, c-Src was detected in c-Cbl immune complexes prepared from OCL lysates (Fig. 2*a*; anti-Src) and in reprecipitates from c-Cbl immunoprecipitated after kinase assay (not shown). Reciprocally, c-Cbl could be immunoprecipitated from c-Src immunoprecipitates after *in vitro* kinase assay (Fig. 2*b*). This association was not always detectable, however, possibly owing to variations in the levels of activated c-Src. In the absence

of a specific stimulus, Src was not detected in c-Cbl immunoprecipitates from fibroblasts overexpressing c-Src, despite the high levels of Src expression in these cells (Fig. 2*a*). In v-Src-transformed fibroblasts, association of Src and Cbl was not detected either (Fig. 2*a*), although we have reported weak association under mild detergent lysis conditions⁶. *In vitro*, and as shown in Fig. 2*c* (left), c-Cbl consistently associated with the SH3 domain of c-Src, as found for other Src-family members^{6,9}, probably binding to the proline-rich region of c-Cbl (ref. 16). Thus, despite some experimental variability, it is likely that c-Src and c-Cbl associate *in vivo*: on the one hand, this interaction between enzyme and substrate may be very transient; on the other hand, the absence of any tyrosine-kinase activity in c-Cbl immunoprecipitates from Src⁻ OCLs suggests that c-Cbl phosphorylation is c-Src-dependent even if the two proteins do not directly interact, and that c-Cbl is downstream of c-Src in OCLs.

As would be predicted from the cell specificity of the defect in the c-src knockout mice, the results in OCLs are in contrast with the findings in other cell types. In osteoblasts or fibroblasts, c-Cbl is present but not constitutively tyrosine-phosphorylated or associated with a kinase (Fig. 1*b*), whereas in fibroblasts, c-Cbl can be phosphorylated in response to PDGF in both Src⁺ and Src⁻ cells (Fig. 1*c*). Together these results strongly suggest that c-Src (or a c-Src-dependent kinase) is the predominant tyrosine kinase which phosphorylates c-Cbl in OCLs, and that this function cannot be compensated by other members of the Src family of non-receptor tyrosine kinases in this cell type. These features are consistent with the osteoclast-specific dysfunction observed in c-src-deleted mice.

If c-Cbl and c-Src are on the same signalling pathway in OCLs, their subcellular localizations may partially overlap in these cells and c-Cbl localization might differ between wild-type and Src⁻ osteoclasts and OCLs. Confocal microscopy revealed that, as we reported for c-Src (ref. 2), c-Cbl localized to punctate vesicular-like structures in OCLs. Although most vesicles contained exclusively c-Cbl or c-Src, the two colocalized in ~20% of these structures (Fig. 3*a, b*). Most of the double-labelled structures

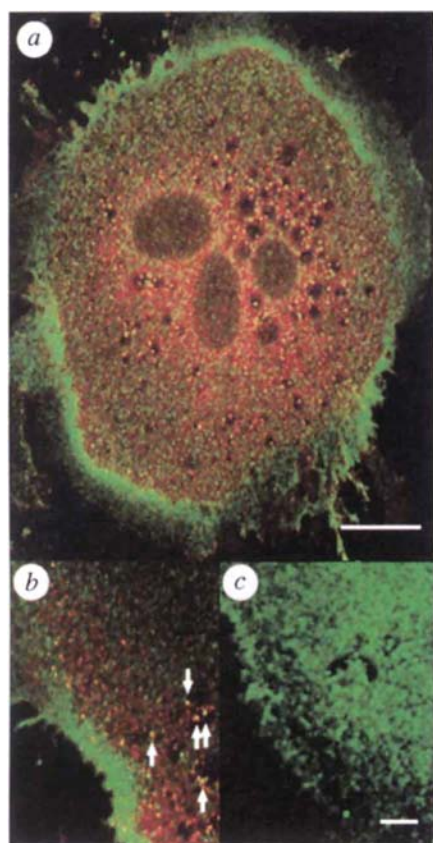


FIG. 3 Double-immunofluorescence staining of c-Cbl and c-Src in OCLs. *a*, Co-staining with anti-Src (red) and anti-c-Cbl antibody (green); *b*, at higher magnification. Note the localization of c-Cbl to the periphery (green) and the co-localization of c-Src and c-Cbl (yellow) on intracellular vesicular structures indicated by arrows. *c*, Immunolocalization of c-Cbl in Src⁻ OCLs. Note the lack of c-Cbl positive vesicular structures in the cell periphery and the accumulation of c-Cbl toward the centre of Src⁻ OCLs. Scale bars, 5 µm (*a*) and 1 µm (*b* and *c*).

were seen in the perinuclear areas, where Golgi complexes and abundant transport vesicles are present^{17,18}, and in the immediate vicinity of, and often in direct contact with, large vacuoles within the cells. Such localizations suggest that both c-Cbl and c-Src associate with vesicular structures in regions active in endocytosis and/or exocytosis and may therefore be involved in some aspects of vesicular trafficking, consistent with our observations on the translocation of c-Cbl in other cell types⁶. One specific population of such vesicular structures, which stained positively for c-Cbl but not for c-Src, was concentrated at the cell periphery in Src⁺ OCLs (green in Fig. 3*a, b*) or in OCLs treated with c-src sense oligonucleotides (data not shown). This localization of c-Cbl-positive structures was not seen in Src⁻ OCLs (Fig. 3*c*) or in c-Src⁺ OCLs treated with c-Src antisense oligonucleotides (data not shown), where the c-Cbl-positive vesicles seemed to accumulate in larger numbers towards the centre of the OCLs. Similar observations were made in authentic osteoclasts from c-Src⁺ and c-Src⁻ animals (not shown). Thus c-Src may be required for c-Cbl to localize in this region of the cell. As c-Src is also required for the tyrosine phosphorylation of c-Cbl in OCLs (Fig. 1*a*), either c-Src-dependent phosphorylation of c-Cbl or its association with c-Src, whether direct or within a multimolecular complex, may be required for the targeting of some vesicles to the peripheral cytoskeleton of OCLs, or otherwise c-Cbl may associate with the vesicles in this region only when it is tyrosine-phosphorylated.

These results all pointed to a functional role for c-Cbl in osteoclasts. To establish the functional link between c-Cbl and c-Src, as well as between c-Cbl and bone resorption, c-cbl or c-src

control (sense, scrambled and inverted) and antisense oligonucleotides were added to OCLs in co-cultures that had been plated either on plastic dishes for analysis of protein expression or on dentine slices for measurement of bone resorption. Figure 4*a* shows that c-cbl antisense oligonucleotides markedly and specifically depleted the cells of c-Cbl protein. The levels of c-Src (not shown), of the cytoskeletal proteins actin (Fig. 4*a*) and vinculin (not shown), or of known c-Src substrates including cortactin, p85 PI(3)K (Fig. 4*a*), or of FAK, p120 and p130Cas (not shown) were not affected. In parallel, the ability of OCLs to resorb bone was markedly decreased by c-cbl antisense (by $76.2 \pm 4\%$, $P < 0.001$), an inhibition comparable to that achieved by c-src antisense ($77 \pm 1\%$, $P < 0.001$; Fig. 4*b*), but the number of adherent OCLs remained unchanged (data not shown). Sense, inverted and scrambled c-cbl oligonucleotides had no effect on c-Cbl or other proteins levels, or on bone resorption (Fig. 4*a*). Significantly, both bone resorption (Fig. 4*b*) and c-Cbl tyrosine-phosphorylation, but not c-Cbl protein levels (Fig. 4*c*), were

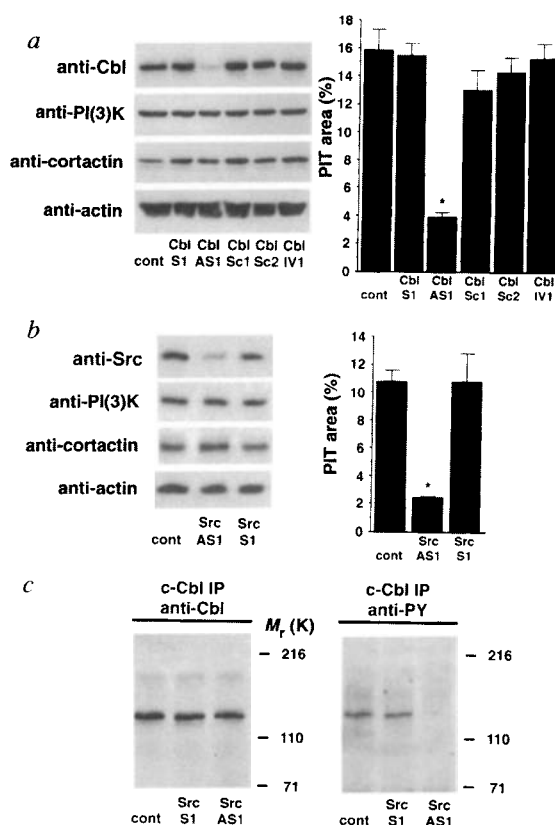


FIG. 4 Antisense c-src oligonucleotides block c-Cbl phosphorylation and bone resorption, whereas c-cbl antisense oligonucleotides block bone resorption. *a*, Western blot analysis of OCL total lysates blotted with antibodies to c-Cbl and reblotted with antibodies to p85 PI(3)K, cortactin and F-actin. c-cbl-antisense oligonucleotides (AS1) depleted the OCLs of c-Cbl, whereas other proteins or c-Src substrates were not affected. Sense (S1), scrambled (SC1, SC2) and inverted (IV1) oligomers had no effects. The resorption pit assay shows that treatment of the cells with antisense inhibits the resorbing activity of the OCLs, and control oligonucleotides had no effect (asterisk, $P < 0.001$). *b*, In a similar analysis, c-src antisense oligonucleotides depleted the OCLs of Src protein and also inhibited bone resorption (asterisk, $P < 0.001$). The number of OCLs formed and present at the end of the culture was not affected by any of the oligomers used (data not shown). The results shown here were reproduced in three independent experiments. *c*, c-src antisense oligonucleotides inhibit c-Cbl tyrosine phosphorylation. c-Cbl immunoprecipitates (c-Cbl IP) from OCLs treated with either c-src control oligonucleotides, c-src antisense oligonucleotides or controls without treatment, were immunoblotted with anti-phosphotyrosine antibody (anti-PY). The blot was then stripped of antibody and reprobed with anti-c-Cbl antibody to confirm that equal amounts of c-Cbl were loaded in each lane.

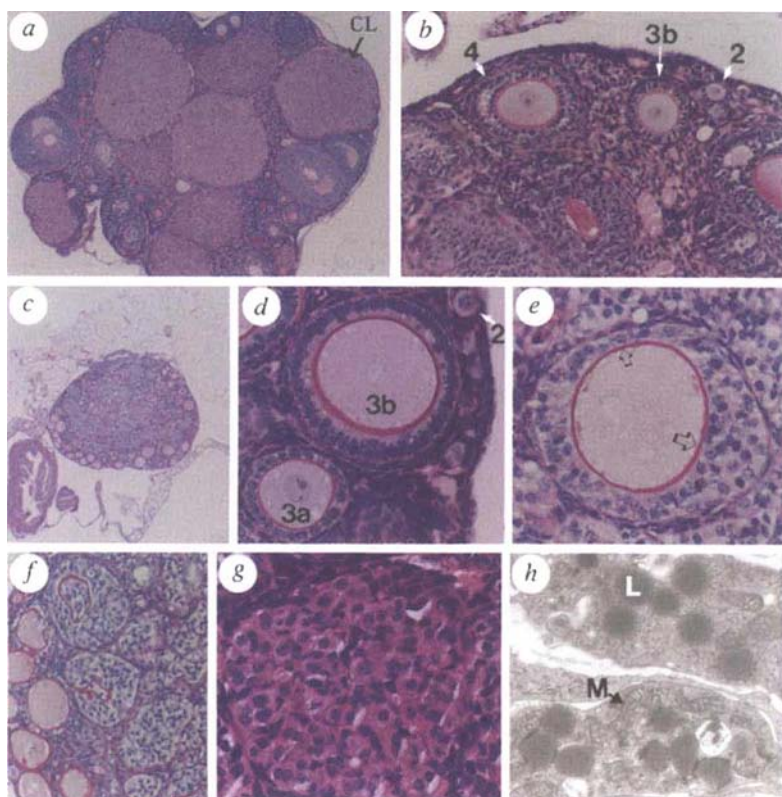
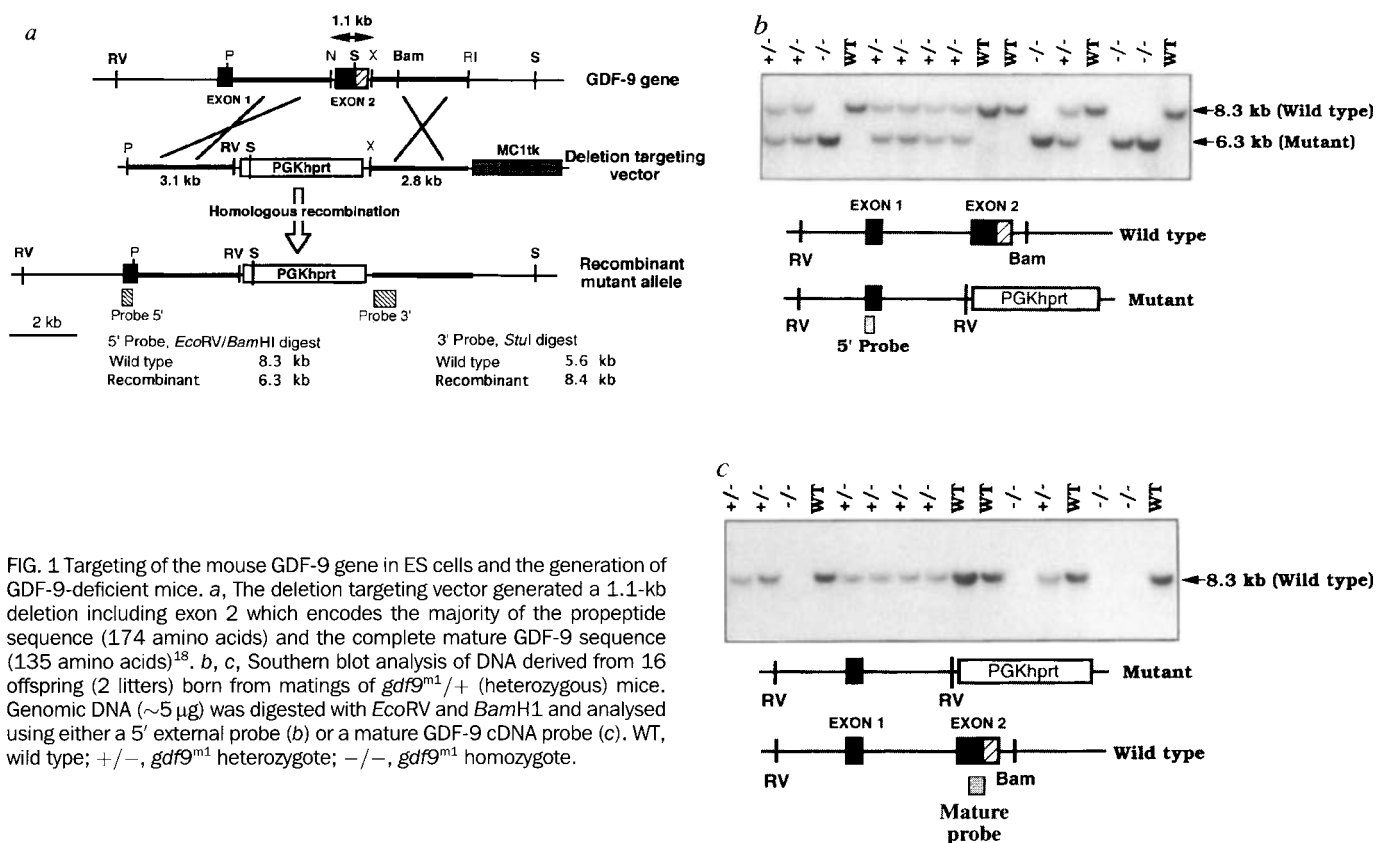


FIG. 2 Histological analysis of adult ovaries. **a**, **b**, Normal follicular development is seen in control (*gdf9*^{m1}/+) ovaries including corpora lutea (CL) and primordial (type 2), one-layer (type 3b), and two-layer (type 4) follicles using the previously described classification²⁷. **c**–**g**, Analysis of ovaries from *gdf9*^{m1}/*gdf9*^{m1} mice. Primary one-layer follicles are present around the periphery of the ovary (**c**) and many primordial (type 2), one-layer growing (type 3a) and one-layer primary (type 3b) follicles appear normal by light microscopy (**d**). Abnormal follicles (**e**) demonstrate asymmetrical granulosa cell growth (small arrow, one layer; large arrow, several layers). After oocyte degeneration, granulosa cells become vacuolated (**f**) or occasionally appear luteinized (**g**). Electron microscopic analysis (**h**) of a follicle-like cell shows mitochondria (M) and lipid (L) droplets. Magnifications: **a**, **c**: ×18; **b**, **f**: ×90; **d**, **e** and **g**: ×7,000.

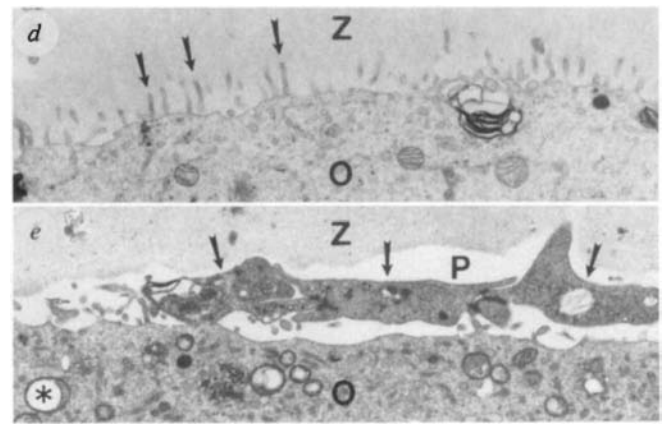
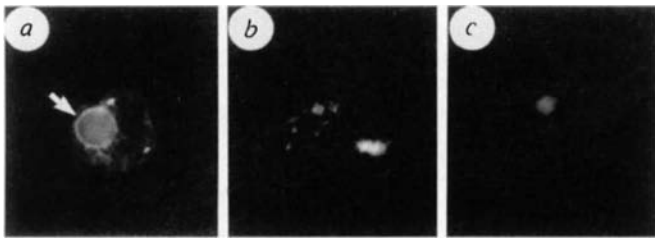


FIG. 3 Fluorescence (a–c) and electron (d, e) micrographs of control (b) and *gdf9^{m1}/gdf9^{m1}* oocytes (a, c, d, e). a, Intrafollicular oocytes exhibit chromatin rims around the nucleolus (arrow) confined to a perinuclear location. b, c, Cultured control (*gdf9^{m1/+}*) oocytes undergo meiotic maturation to metaphase-2 (b) whereas many *gdf9^{m1}/gdf9^{m1}* oocytes undergo germinal vesicle breakdown and chromatin condensation but remain arrested at this stage (c). d, Before the transformation of follicles to a luteinized phenotype, oocytes (O) are surrounded by zona pellucida (Z) and microvilli (arrows) extend into the zona pellucida. e, In luteinized follicles, microvilli are withdrawn from the zona pellucida, three follicle-

cell projections (arrows) occupy the perivitelline (P) space, and mitochondria (asterisk) are often vacuolated. Magnifications: a–c, $\times 300$; d, e, $\times 7,000$.

and fertile and were intercrossed to obtain *gdf9^{m1}/gdf9^{m1}* mice (GDF-9-deficient). Genotype analysis of 200 F₂ offspring from these intercrosses (Fig. 1b, c) was consistent with a normal mendelian ratio of 1:2:1 (48 wild type (24%), 102 heterozygotes (51%) and 50 homozygotes (25%)) and a similar number of male and female homozygotes were produced (23 male, 27 female). Consistent with the female-specific expression of GDF-9 mRNA, *gdf9^{m1}/gdf9^{m1}* male mice are fertile. In contrast, matings of twenty *gdf9^{m1}/gdf9^{m1}* female hybrid (129SvEv/C57BL/6) and 129SvEv inbred mice with control wild-type males during a 6–11 month period, failed to produce any offspring. Thus, in contrast to *gdf9^{m1}/gdf9^{m1}* males and *gdf9^{m1}/+* females, *gdf9^{m1}/gdf9^{m1}* females are infertile.

To determine the causes of the infertility in *gdf9^{m1}/gdf9^{m1}* females, ovaries were examined morphologically and histologically. Ovaries from *gdf9^{m1}/gdf9^{m1}* mice were smaller than controls from as early as one week of age when two-layer primary follicles are initially seen in the controls (Fig. 2, and data not shown). In contrast to the normal stages of folliculogenesis and the presence of corpora lutea in adult control ovaries (Fig. 2a, b), ovaries from *gdf9^{m1}/gdf9^{m1}* mice lack corpora lutea and fail to demonstrate any normal follicles beyond the primary one-layer follicle stage (Fig. 2c). Numerous primordial and early primary one-layer follicles are evident in *gdf9^{m1}/gdf9^{m1}* ovaries, and oocytes found within primary one-layer follicles achieve normal size and form zona pellucida (Fig. 2c, d). However, in contrast to control ovaries (Fig. 2b), *gdf9^{m1}/gdf9^{m1}* ovaries never contain follicles with two layers of granulosa cells surrounding the oocyte; follicles beyond the one-layer follicle stage in the *gdf9^{m1}/gdf9^{m1}* ovaries are abnormal and contain an asymmetrical arrangement of atypical 'granulosa' cells (Fig. 2e). This figure shows the most advanced follicle observed (a structure in which granulosa cells surround an oocyte). A thecal layer is also conspicuously absent in all primary one-layer follicles found in *gdf9^{m1}/gdf9^{m1}* ovaries, as determined by light and electron microscopy. In addition, oocyte degeneration is apparent in follicle-like structures that contain collapsed zona pellucida (magenta-stained structures), and vacuolated granulosa cells are present both outside and within the zona pellucida remnant (Fig. 2f). Granulosa cells found in these abnormal follicle-like structures exhibit signs of steroidogenic activity (Fig. 2f, g), as evidenced by the presence of numerous lipid droplets and mitochondria with tubular cristae (Fig. 2h) and the dilated appearance of *gdf9^{m1}/gdf9^{m1}* uteri (data not shown). Surprisingly, in contrast to control ovaries, follicular atresia (follicles in which cells are undergoing apoptosis) is never observed in ovaries from *gdf9^{m1}/gdf9^{m1}* mice. Thus, the cells of these follicle-like structures are not normal granulosa cells, and the block in folliculogenesis caused by GDF-9 absence occurs before granulosa cells normally gain the competence to undergo atresia.

At the ultrastructural level, several additional abnormalities in follicle organization and oocyte structure were apparent (Fig. 3). Within fully grown oocytes surrounded by zona pellucida, oocyte microvilli extend into the zona pellucida and extensions from granulosa cells were observed within the perivitelline space (Fig. 3d). Oocyte microvilli were withdrawn from the zona pellucida in other follicles where granulosa cell projections envelop oocytes (Fig. 3e). Despite achieving full size, oocytes from *gdf9^{m1}/gdf9^{m1}* mice rarely contained cortical granules, and most organelles were clustered around the germinal vesicle. However, most full-grown oocytes exhibit other normal ultrastructural features, including vacuolated mitochondria, dispersed Golgi complexes, and abundant fibrous lattices (data not shown). Thus, consistent with the expression of GDF-9 mRNA, which is first detected in oocytes of primary one-layer follicles, *gdf9^{m1}/gdf9^{m1}* female mice exhibit a block in folliculogenesis at this stage. Absence of GDF-9 prevents formation of normal two-layer follicles, leads to eventual oocyte degeneration, and causes the follicle cells to 'differentiate'.

To study the ovarian defects in more detail, oocytes from 10–12-week-old mice were collected and analysed. Oocytes obtained from *gdf9^{m1}/gdf9^{m1}* females ($71 \pm 4 \mu\text{m}$) were slightly larger than *gdf9^{m1/+}* controls ($66 \pm 4 \mu\text{m}$). Furthermore, germinal vesicle oocytes from control mice exhibited predominantly rimmed chromatin configurations (10/13, 77%), and after culturing, 86% of oocytes resumed meiosis progressing to either metaphase 1 (9/35, 26%) or metaphase 2 (21/35, 60%) (Fig. 3b). Rimmed chromatin configurations were also seen in *gdf9^{m1}/gdf9^{m1}* germinal vesicle stage oocytes (47/67, 70%) (Fig. 3a), but upon culture seemed to be defective in their meiotic competence (Fig. 3c). Only 40% (20/50) of oocytes proceeded to metaphase 1 (8/50) or metaphase 2 (12/50), 26% retained germinal vesicles (13/50), whereas the remaining 34% (17/50) exhibited abnormal germinal vesicle breakdown as evidenced by chromatin aggregates and a failure to form meiotic spindles. Thus, although *gdf9^{m1}/gdf9^{m1}* oocytes undergo some normal transitions in chromatin organization^{19,20}, meiotic competence acquisition of the oocytes is impaired.

In addition to these abnormalities, ovaries from 25–31-week-old *gdf9^{m1}/gdf9^{m1}* females contain either single unilateral (5/14) or bilateral (1/14) ovarian follicular cysts (Fig. 4a, b). These cysts (only observed in mutant ovaries) are lined by several layers of flattened granulosa cells (Fig. 4b, and data not shown). As follicular cysts are often seen with hypergonadotropism (especially increased levels of luteinizing hormone (LH))²¹, we determined the serum follicle-stimulating hormone (FSH) and LH levels by radioimmunoassay. In contrast to wild-type and *gdf9^{m1/+}* females, serum FSH and LH levels in the *gdf9^{m1}/gdf9^{m1}* females were elevated threefold and twofold, respectively (Fig. 4c, d). Thus, these mice are hypogonadal and hypergonadotropic. Despite the elevated serum FSH and LH levels, LH

receptor, FSH receptor and activin type II receptor mRNA levels seem unaltered in the *gdf9^{ml}/gdf9^{ml}* ovaries, and cytochrome P450 aromatase mRNA levels are 50% reduced (Fig. 4e). In addition, ovaries of 3-week-old *gdf9^{ml}/gdf9^{ml}* mice are unresponsive to pregnant-mare serum gonadotropin/human chorionic gonadotropin superovulation treatment (data not shown). These results indicate that signals downstream of the FSH and LH receptors are altered in the absence of GDF-9 leading to altered negative feedback by gonadal steroids and peptides.

The *gdf9^{ml}/gdf9^{ml}* ovarian phenotype raises several interesting questions with respect to the coordination of oogenesis and folliculogenesis. Although some aspects of oogenesis proceed normally²², *gdf9^{ml}/gdf9^{ml}* oocytes fail to produce a normal complement of cortical granules, do not undergo dispersion of intracellular organelles and acquire limited meiotic competence. If the completion of oocyte growth and differentiation requires continued follicle cell support^{6,23}, then the characteristic *gdf9^{ml}/gdf9^{ml}* oocyte phenotype may be secondary to incomplete or failed somatic cell input during the later stages of oogenesis. These findings would implicate GDF-9 in sustaining the growth and differentiation status of the follicle after the primary one-layer follicle stage. The failure to develop a well-defined thecal layer may be coupled to this important transition and further evidences the complexity of gamete and somatic cell interplay during normal ovarian follicular development. □

Methods

Targeted deletion. Sequence (>20 kb) encompassing the 2-exon mouse GDF-9 gene¹⁸ was isolated from a mouse 129SvEv genomic library. Linearized vector (25 µg) was electroporated into the *hprt*-negative AB2.1 ES cell line, cell clones were selected in HAT (hypoxanthine, aminopterin, and thymidine) and FIAU (1-(2'-deoxy-2'-fluoro-β-D-arabinofuranosyl)-5-iodouracil), DNA from the clones was analysed by Southern blot, and targeted ES cell clones were expanded and injected into blastocysts as described^{10,24,25}. Enrichment in HAT and FIAU was 6.8-fold compared with HAT alone. The recombinant deleted allele can be distinguished by Southern blot analysis using 5' and 3' probes (Fig. 1). 83% of ES cell clones (303 of 367) screened demonstrated the correct GDF-9 mutation. Germline transmission of the deleted GDF-9 allele was demonstrated from chimaeras derived from 3 independent ES cell clones. The phenotypes for homozygotes derived from these 3 independent ES cell clones were identical. The presence of a single 6.3-kb fragment in four of the lanes in Fig. 1b indicates a homozygous mutant genotype. Absence of a hybridizable signal in the lanes genotyped as homozygotes when using the mature GDF-9 probe (Fig. 1c) confirmed that the mutant *gdf9^{ml}* allele is a null allele.

Histological analysis. Periodic acid Schiff/haematoxylin stain was used as described^{10,12}. Electron microscopic analysis was done on ovaries fixed in 3% glutaraldehyde, 11% paraformaldehyde in 0.1M cacodylate buffer, pH 7.4, followed by 1% osmium tetroxide. Plastic sections were stained with uranyl acetate and lead citrate before viewing.

Oocyte sizing. Oocytes (*n* = 21) from *gdf9^{ml}/+* mice were stripped of cumulus cells, and those (*n* = 22) from *gdf9^{ml}/gdf9^{ml}* mice either lacked adherent granulosa cells or retained a single layer of granulosa cells. Germinal vesicle chromatin patterns in oocytes fixed immediately after isolation were evaluated and stained with Hoechst 33258 and classified as described¹⁹. To assess the meiotic competence, oocytes were cultured for 16 h before fixation and staining. Germinal vesicle and meiotic competence experiments were done on 4–10-week-old mice.

Radioimmunoassays. Reagents from the National Hormone and Pituitary Distribution Program were used as described^{10,12}. Serum from 6–9 mice per genotype was used for the analyses.

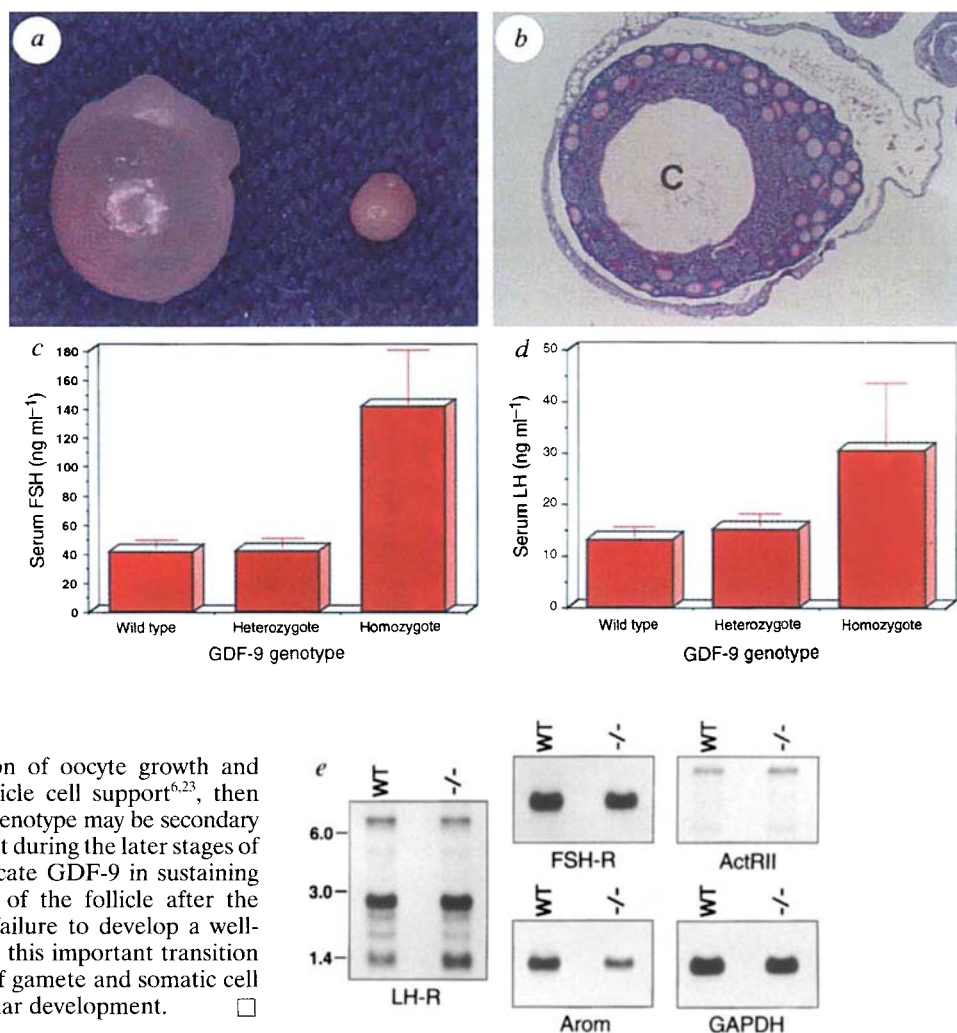


FIG. 4 Morphological and histological analysis of ovarian cysts, hormonal analysis of wild-type and mutant mice, and northern blot analysis of ovarian RNA. **a**, Gross analysis of a 1-cm clear fluid-filled follicular cyst (left) and the contralateral ovary (right) from a GDF-9-deficient mouse. **b**, Histological analysis of an ovary from a *gdf9^{ml}/gdf9^{ml}* mouse which contained a small cyst (magnification, $\times 36$). **c**, **d**, Serum FSH (**c**) and LH (**d**) levels (mean $\pm$ s.e.m.) in adult female randomly cycling wild-type, *gdf9^{ml}/+* (heterozygote), and *gdf9^{ml}/gdf9^{ml}* (homozygote) mice. **e**, Ovarian mRNA isolated from wild-type (WT) or *gdf9^{ml}/gdf9^{ml}* ($-/-$) mice was probed with mouse LH receptor (LH-R), FSH receptor (FSH-R), activin receptor type II (ActRII), cytochrome P450 aromatase (Arom), or GAPDH cDNA probes.

Northern blot analysis. Total RNA was extracted from ovaries from 19 *gdf9^{ml}/gdf9^{ml}* or wild-type females using RNA STAT-60 as described by the manufacturer (Leedo Medical Lab, Houston, Texas). Poly(A)⁺ RNA was purified using Oligotex-d7 beads according to the manufacturer's instructions (QIAGEN Inc., Chatsworth, California). Poly(A)⁺ RNA (10 µg) was electrophoresed on a 1.5% agarose, 7.6% formaldehyde gel, and transferred to Hybond-N (Amersham) nylon membrane. The membrane was sequentially hybridized, washed, exposed and stripped for the next hybridization as described²⁶. The complementary DNA probes were cloned and sequenced in our laboratory using standard techniques. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA was used as an internal control.

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CORRESPONDENCE and requests for materials should be addressed to M.M.M. (e-mail: mmatzuk@bcm.tmc.edu).

Pain responses, anxiety and aggression in mice deficient in pre-proenkephalin

Monika König, Anne M. Zimmer, Heinz Steiner*†, Philip V. Holmes†‡, Jacqueline N. Crawley‡, Michael J. Brownstein & Andreas Zimmer

Unit on Developmental Biology, Laboratory of Cell Biology, * Section on Neuroanatomy, Laboratory of Neurophysiology, and ‡ Section on Behavioural Neuropharmacology, Experimental Therapeutics Branch, National Institute of Mental Health, Bethesda, Maryland 20892, USA

ENKEPHALINS are endogenous opioid peptides that are derived from a pre-proenkephalin precursor protein^{1,2}. They are thought to be vital in regulating many physiological functions, including pain perception and analgesia, responses to stress, aggression and dominance^{3–5}. Here we have used a genetic approach to study the role of the mammalian opioid system. We disrupted the pre-proenkephalin gene using homologous recombination in embryonic stem cells to generate enkephalin-deficient mice. Mutant *enk^{-/-}* animals are healthy, fertile, and care for their offspring, but display significant behavioural abnormalities. Mice with the *enk^{-/-}* genotype are more anxious and males display increased offensive aggressiveness. Mutant animals show marked differences from controls in supraspinal, but not in spinal, responses to painful stimuli. Unexpectedly, *enk^{-/-}* mice exhibit normal stress-induced analgesia. Our results show that enkephalins modulate responses to painful stimuli. Thus, genetic factors may contribute significantly to the experience of pain.

The enkephalin gene was mutated to block enkephalin peptide production by replacing the 5' part of exon 3, which contains all enkephalin-coding sequences¹, with PGK-*neo* (Fig. 1a). Chimaeric

mic mice were generated with two targeted embryonic stem (ES) cell lines, one of which transmitted the mutant allele. This ES cell clone had an unexpected partial duplication of exon 3 sequences (Fig. 1a). Homozygous *enk^{-/-}* mice (Fig. 1b) were obtained with the expected mendelian frequency. Northern-blot analysis of brain and testis tissues showed that messenger RNA levels were reduced by about 50% in heterozygous *enk^{+/-}* mice and virtually absent in homozygous *enk^{-/-}* animals (Fig. 1c). Opioid peptide levels in striatal brain extracts were determined by radioimmunoassays (RIA). As expected, no enkephalin immunoreactivity was detected in *enk^{-/-}* mice and enkephalin levels were reduced by 44% in *enk^{+/-}* mice. No differences were found in dynorphin and endorphin peptide levels between the different genotypes (Fig. 1d).

Homozygous *enk^{-/-}* animals appeared healthy, were of similar size and weight as wild-type littermates, and had no gross defects. No morphological abnormalities were found on close examination of serial brain sections or in other tissues. Mutant mice were fertile and cared for their offspring, but they behaved abnormally. The abnormal behaviours in *enk^{-/-}* pups and adolescent mice included hiding under the bedding, frantic running or jumping, and prolonged freezing in response to moderate noise or movements. Adult mice were sometimes aggressive, attacking caretakers and investigators.

Opiates have profound analgesic effects. It has been suggested that the endogenous opioid system might be important in modulating reaction to painful stimuli, in situations where environmental danger is perceived (stress-induced analgesia)⁶. Enkephalinergic neurons are present in many regions that mediate pain responses, including the mesencephalic central grey matter and the substantia gelatinosa in the spinal cord^{2,7}.

We tested pain responses and stress-induced analgesia in *enk^{-/-}* mice in several ways. First, we used the tail-flick method to assess pain sensitivity and stress-induced analgesia mediated at the spinal level⁸. Pre-stress tail-flick latencies were identical in *enk^{-/-}* and *enk^{+/-}* mice (Fig. 2a), indicating that *enk^{-/-}* animals exhibit a normal pain response at the spinal level. The mice were then subjected to repeated foot shocks, a procedure that induces an opioid-dependent form of analgesia in the tail-flick assay⁸. As shown in Fig. 2a, mice of both genotypes exhibited a similar increase in the tail-flick latency, indicating that similar levels of analgesia were induced.

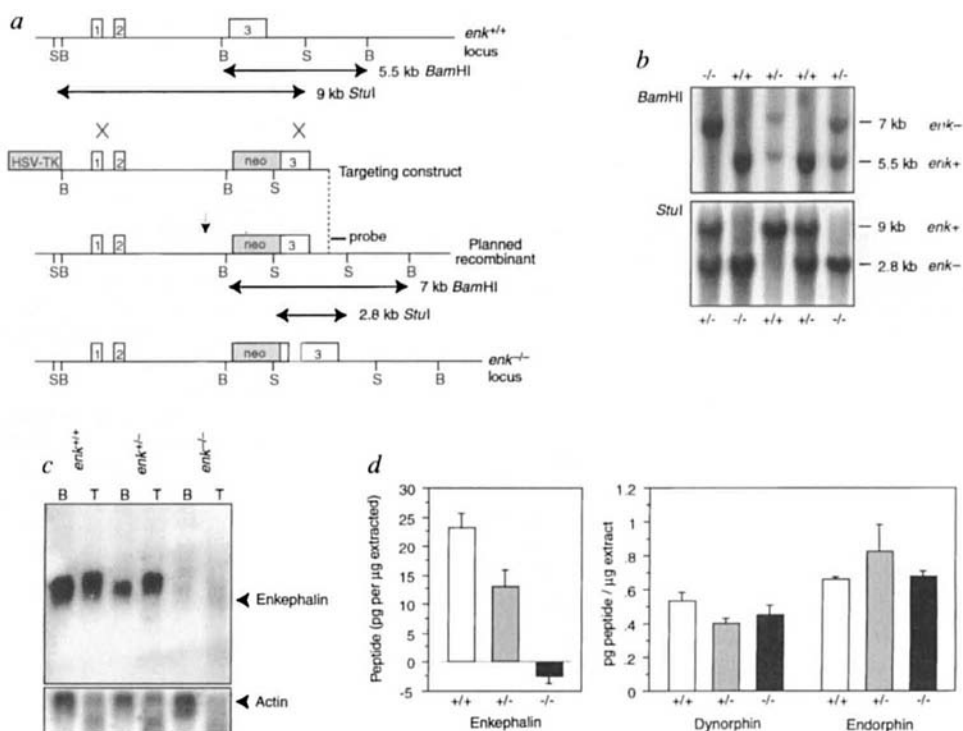
Second, the hot-plate assay^{9,10} was used to measure pain responses and analgesia mediated by supraspinal mechanisms. Mutant (*enk^{-/-}*) mice vigorously shook their hind paws immediately after being placed on the hot surface. In contrast, *enk^{+/-}* mice first explored the new environment and rarely shook their hind paws. As depicted in Fig. 2b, *enk^{-/-}* mice showed a significantly shorter jump latency than *enk^{+/-}* mice. Thus, in contrast to the spinal threshold, the supraspinal pain threshold is lower in *enk^{-/-}* mice, and enkephalins are involved in the modulation of such pain responses.

Analgesia can be induced by stressing the mice with a short swim. Whether this analgesia is mediated by opioid or non-opioid systems is dependent on the water temperature^{9,10}. A forced swim in 34 °C water induces minimal analgesia that seems to be entirely opioid dependent, as it can be blocked with the opioid antagonist naloxone (Fig. 2c). Mice of *enk^{-/-}* and *enk^{+/-}* genotypes exhibited identical levels of analgesia in this test, indicating that this form of analgesia, although opioid in nature, is not mediated by enkephalins. A forced swim in 4 °C water induces analgesia that has an opioid-dependent and an opioid-independent component, as it is only partially reversible by high doses of naloxone (Fig. 2d). Again, *enk^{-/-}* and *enk^{+/-}* mice exhibited nearly identical levels of analgesia under these conditions. Thus, enkephalins are not critically involved in stress-induced analgesia, contrary to previous expectations^{6,11}.

To evaluate the effects of the enkephalin mutation on pain responses in more detail, we used a formalin test¹². Formalin injections into the hind paw produce a nociceptive stimulus by

† Present addresses: Department of Anatomy and Neurobiology, University of Tennessee, College of Medicine, Memphis, Tennessee 38163, USA (H.S.); Department of Psychology, University of Georgia, Athens, Georgia 30602, USA (P.V.H.).

FIG. 1 Targeted mutagenesis and expression analysis of *enk*. **a**, Restriction map of the wild-type *enk* locus, the targeting construct, the planned recombination, and the *enk*^{-/-} locus. The 3' external probe used for Southern blot analysis and the expected restriction fragments are indicated. S, *Stu*I; B, *Bam*HI. **b**, Genotyping of offspring from *enk*^{+/-} × *enk*^{+/-} matings by Southern blot analysis of *Bam*HI- and *Stu*I-digested mouse tail DNA. **c**, Northern blot analysis of expression of enkephalin mRNA in brain (B) and testis (T) of *enk*^{+/-}, *enk*^{+/-} and *enk*^{-/-} mice. Size difference of *enk* transcripts in brain and testis tissues is caused by differential promoter usage. Blot was secondarily developed with a β -actin-specific probe. **d**, Met-enkephalin, dynorphin and endorphin peptide levels (mean ± s.e.m.) in striatal extracts as determined by radioimmunoassay³⁰ from four animals of each genotype.



inducing tissue damage, thus providing a model for inescapable pain. After injection of formalin, *enk*^{-/-} and *enk*^{+/-} animals show very different responses. Most *enk*^{+/-} animals showed pain responses, such as paw lifting and licking, within a minute of the injection. In contrast, these responses were significantly reduced in *enk*^{-/-} animals (Fig. 2e). Some animals never licked or lifted their hind paws at all. However, *enk*^{-/-} mice often vigorously shook the affected hind paw immediately after the injection, some appeared highly agitated, and most animals began to sniff intensely within a few minutes.

To test whether the altered pain responses in *enk*^{-/-} animals are a direct effect of the lack of enkephalin, we injected wild-type CD1 mice with 10 mg per kg body weight naloxone or saline vehicle 10 min before the formalin injection. The naloxone-injected mice exhibited the same behavioural changes as the *enk*^{-/-} mice and a similar reduction in paw lifting and licking (Fig. 2e), indicating

that these behavioural alterations are a direct consequence of the lack of opioid peptides and are not due to adaptive changes in the mutants, or their genetic background¹³.

Locomotor activity of enkephalin-deficient mice was assayed in 'open-field' tests. Mice with the *enk*^{-/-} genotype displayed significantly reduced levels of horizontal activity in an open space, compared with *enk*^{+/-} littermates (Fig. 3a). Habituation was similar in *enk*^{+/-} and *enk*^{-/-} mice, as indicated by the parallel decrease in activity during the course of the 15-min trial (data not shown). These findings are important for interpreting the results of the hot-plate test, as this test measures a motor response to a noxious stimulus. The behaviour of the *enk*^{-/-} animals in the open-field test indicates that the reduced jump latency in *enk*^{-/-} mice is not the result of generally increased activity in an unfamiliar environment.

On the other hand, it is unlikely that the reduced locomotor

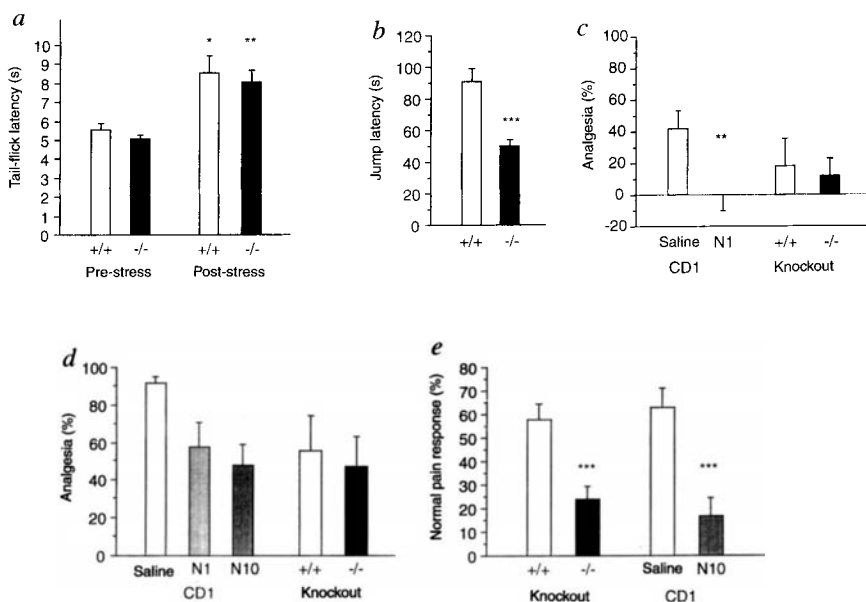


FIG. 2 Pain sensitivity and stress-induced analgesia. **a**, Tail-flick latency (mean ± s.e.m.) before and after foot-shock stress (*enk*^{+/-}, *n* = 8; *enk*^{-/-}, *n* = 11). **b**, Baseline jump latency in the hot-plate assay (*enk*^{+/-}, *n* = 34; *enk*^{-/-}, *n* = 34). **c**, Analgesia induced by a 3-min forced swim in 34 °C water in CD1 control mice injected with saline (*n* = 10) or 1 mg per kg naloxone (N1; *n* = 10) and in *enk*^{-/-} (*n* = 10) and *enk*^{+/-} (*n* = 9) mice. **d**, Analgesia induced by a 90-s swim in 4 °C water in CD1 mice injected with saline (*n* = 10), or 1 mg per kg naloxone (N1; *n* = 10), or 10 mg per kg naloxone (N10; *n* = 10) and in *enk*^{+/-} (*n* = 9) and *enk*^{-/-} (*n* = 9) animals. **e**, Normal pain responses (paw licking and paw lifting) after formalin injection into the hind paw of knockout (*enk*^{+/-}, *n* = 12; *enk*^{-/-}, *n* = 15) and CD1 control mice injected with saline (*n* = 10) or 10 mg per kg naloxone (N10; *n* = 10). Data were analysed with the Wilcoxon signed rank (a) or the Mann-Whitney U-test (b-e). *, *P* < 0.05; **, *P* < 0.01; ***, *P* < 0.001.

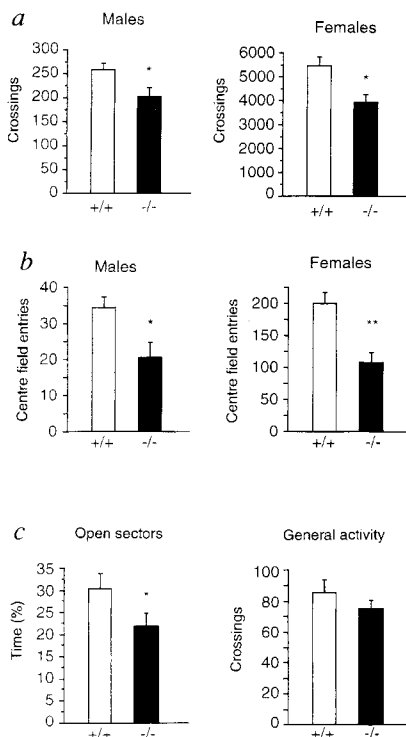


FIG. 3 The open-field assay. Horizontal activity (mean $\pm$ s.e.m.) (a) and centre field entries (b) were significantly lower in $enk^{-/-}$ mice. Note that males ($enk^{+/+}$, $n = 13$; $enk^{-/-}$, $n = 10$) and females ($enk^{+/+}$, $n = 7$; $enk^{-/-}$, $n = 10$) were analysed in different open-field systems. c, Behaviour of male mice in the O-maze assay. Time in the open sectors (mean $\pm$ s.e.m.) (a) and general activity (b) during the 4-min tests are shown for $enk^{+/+}$ mice ($n = 13$) and $enk^{-/-}$ mice ($n = 19$). *, $P < 0.05$; **, $P < 0.01$; Mann-Whitney U test.

activity of $enk^{-/-}$ mice is due to motor defects because rearing rates did not differ significantly between the two genotypes, and $enk^{-/-}$ mice performed well in other motor tasks (data not shown). We therefore tested whether the reduced locomotor activity of $enk^{-/-}$ mice in the open field might reflect increased apprehension or anxiety¹⁴⁻¹⁶ by examining another behaviour that is affected by increased fear; the tendency to stay close to walls in the open field (thigmotaxis¹⁷). As shown in Fig. 3b, $enk^{-/-}$ animals showed a greater preference for peripheral areas of the open field and entered the central area significantly less often than normal mice. We also analysed a different set of animals in an established animal model of anxiety; the elevated O-maze¹⁸. Mice with the $enk^{-/-}$ genotype spent significantly less time in the open sectors of the O-maze than $enk^{+/+}$ mice, indicating increased anxiety, whereas overall activity levels were similar in this test (Fig. 3c). Taken together, these results show that $enk^{-/-}$ mice show reduced exploratory activity in an unfamiliar environment, which suggests increased anxiety or heightened vigilance in these animals.

Mice with the $enk^{-/-}$ genotype were often aggressive, especially when housed individually. Aggressive behaviour in $enk^{-/-}$ mice was analysed in the 'resident-intruder' test¹⁹. Resident mice with the $enk^{-/-}$ genotype were significantly more aggressive than $enk^{+/+}$ mice, as indicated by a higher fighting score and a reduced attack latency (Fig. 4). Wild-type animals initially tolerated intrusions into their territory to a certain extent, but became more aggressive with repeated exposure to an intruder¹⁹, whereas $enk^{-/-}$ mice were immediately intolerant of intruders.

A direct role of enkephalins in modulating emotional behaviours is indicated by their widespread distribution throughout the limbic system including the amygdala, the cingulate and entorhinal cortex, the septum and the hypothalamus². These areas are

known to be involved in the regulation of anxiety as well as aggression²⁰⁻²², and a role for opioid peptides in the modulation of these behaviours has been suggested²³⁻²⁶.

Together, the behavioural changes produced by the pre-proenkephalin mutation can be described as an exaggerated response to painful or threatening environmental stimuli. Pain is a highly subjective sensation. Although nociception at the spinal level is not affected by the pre-proenkephalin mutation, the perception of pain seems to be different in $enk^{-/-}$ mice as indicated by altered supraspinal pain responses. Interestingly, studies in humans show that opiates do not change nociceptive thresholds, but rather reduce the subjective feeling of pain⁷. Thus, $enk^{-/-}$ mice may provide an animal model for the investigation of neuronal pathways and processes that mediate the experience of pain. □

Methods

Targeting the pre-proenkephalin gene. The targeting vector was derived from pPNT²⁷ by introducing a 6.2-kb genomic fragment containing exons I and II upstream, and a 1.3-kb fragment containing part of exon III downstream of *neo*. Upon homologous recombination, 54 bp of exon III and parts of the second intron are replaced by the neomycin gene. Detailed restriction mapping and sequencing revealed that the actual $enk^{-/-}$ locus contained a rearrangement with a partial duplication of exon 3 sequences, downstream of the PGK-*neo* gene. Chimaeric mice were generated by morula aggregation or blastocyst injection with two targeted ES cell lines, R1-14 and R1-29. Chimaeric animals were backcrossed with CD1 mice. The R1-14 mutation was transmitted to progeny.

Behavioural studies. All animals were 8 to 16 weeks old and were housed in a temperature- and humidity-controlled vivarium which was kept on a 12 h dark-light cycle (light on 7:00 EST). Animals had free access to food and water. The genotype of the animals was unknown to the investigator during the tests.

The tail-flick assay was performed using an Omnitech Model TF automated tail-flick apparatus (Omnitech, Columbus, Ohio) using standard procedures^{8,28}. Stress-induced analgesia was assessed one week after control tail-flick testing. Mice were individually placed in a converted mouse shuttle box apparatus with a grid floor. A series of 80 1-mA foot shocks of 1-s duration were delivered at 5-s intervals by a Lafayette model 82400 shock generator switched manually with a 10-V power supply. The tail-flick test was repeated immediately after stress.

The hot-plate test was performed using an electronically controlled hotplate analgesia meter (Columbus Instruments) heated to 55 $\pm$ 0.1 $^{\circ}$ C. The cut-off time was 240 s. Stress-induced analgesia was assessed one day after determining the baseline jump latencies. Per cent analgesia was calculated as ((post-swim latency - baseline latency)/(240 - baseline latency)) $\times$ 100. Each mouse swam individually in a 4-l glass beaker filled with 1.5 to 2 l water of the indicated temperature ($\pm$ 0.5 $^{\circ}$ C). After each swim, mice were patted dry and allowed to rest for 2 min (34 $^{\circ}$ C swim) or 10 min (4 $^{\circ}$ C swim), before being placed on the hot plate.

The formalin test was done by injecting 20 μ l of a 15% formalin solution under the dorsal surface of the right hind paw. One minute after the injection, the behaviour was recorded in 5-s intervals for 10 min with the aid of a hypercard program. Paw licking, paw licking, or other behaviours were recorded. Paw licking and paw lifting were combined as normal pain responses and shown as a percentage of the total activity. Control CD1 animals were injected with saline or naloxone 10 min before the assay.

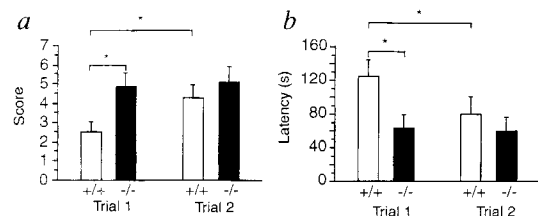


FIG. 4 Aggressive behaviour of mice in the resident-intruder assay. Total fighting scores (mean $\pm$ s.e.m.) (a) and attack latencies (b) for $enk^{+/+}$ ($n = 21$) and $enk^{-/-}$ mice ($n = 20$) in the two 4-min test trials are depicted. $enk^{-/-}$ mice showed a significantly higher fighting score (* $P < 0.05$, Mann-Whitney U test) and a shorter attack latency (* $P < 0.05$) than $enk^{+/+}$ mice in the first test trial. Aggressive behaviour did not differ significantly between the two genotypes in the second test trial. In $enk^{+/+}$ mice, aggressive behaviour increased from trial 1 to trial 2 (score and latency, * $P < 0.05$, Wilcoxon signed rank), but it did not increase significantly in $enk^{-/-}$ animals.

Male behaviour in an open field (60 × 60 cm, with lines dividing the floor into 3 × 3 squares) was videotaped. Rearing events were counted during the experiment, line crossings and central field entries were counted from video tapes. Females were analysed in an Omnitech Digiscan Model RXYZCM8 (Omnitech). Each test session lasted for 15 min.

The O-maze (diameter, 46 cm; runway width, 5.5 cm) consisted of two open and two wall-enclosed sectors of equal size, and was elevated 40 cm above ground. Animals were placed on one open sector in front of an enclosed sector. Animal behaviour was video taped. Time spent in the open sectors and general activity of the animals were evaluated from the tapes. Time in a new sector was measured as soon as the animal entered with four feet. General activity was assessed by counting all entries with at least two feet into a new sector. Rodents tend to avoid the open parts of such runways; this is considered to reflect anxiety^{18,29}.

Resident males were housed individually for about 4 weeks before the experiment. Intruder animals of a similar age were housed in groups of 4 to 5 mice per cage. Animals received two training sessions in the morning and two test sessions in the afternoon, each lasting for four min. All test sessions were video taped. Two measures of aggressiveness were evaluated: attack latency and attack frequency/duration, expressed as a fighting score. To avoid the difficulties and ambiguities associated with counting of individual bites or attacks we developed a time-sampling system to estimate the intensity of fighting. For this purpose, each 4-min test trial was divided into 10-s blocks. Every block with fights scored 1, others 0. All scores were added to give a final fighting score. The analysis was aided through the use of a hypercard program to automate the time sampling.

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CORRESPONDENCE and request for materials and computer programs should be addressed to A.Z. (e-mail: zimmer@codon.nih.gov).

B-cell-specific coactivator OBF-1/OCA-B/Bob1 required for immune response and germinal centre formation

Daniel B. Schubart, Antonius Rolink*, Marie H. Kosco-Vilbois†, Florence Botteri & Patrick Matthias

Friedrich Miescher Institute, Maulbeerstrasse 66, CH-4002 Basel, Switzerland

* Basel Institute for Immunology, Grenzacherstrasse 487, CH-4005 Basel, Switzerland

† Glaxo Institute for Molecular Biology, ch. des Aulx 14, CH-1228 Plan-les-Ouates, Switzerland

THE B-lymphocyte-specific transcriptional factor called Oct binding factor (OBF)-1, OCA-B or Bob1 (refs 1–3) is thought to be involved in the transcription of immunoglobulin genes through recruitment to the highly conserved octamer site of immunoglobulin promoters, mediated by either Oct-1 or Oct-2. To define the *in vivo* role of OBF-1 we have used gene targeting in embryonic stem cells to generate mice lacking the coactivator OBF-1. Such OBF-1^{-/-} mice are born normally, are fertile and seem healthy, and surprisingly, rearrangement and transcription of immunoglobulin genes are largely unaffected. However, mice deficient in OBF-1 have reduced numbers of mature B cells and a severe reduction in the number of recirculating B cells, but otherwise show normal B-cell differentiation. Serum IgA and particularly IgG levels are greatly reduced. If mutant mice are immunized with either a thymus-independent or a thymus-dependent antigen, their immune responses are dramatically weakened. Strikingly, germinal centres completely fail to develop after immunization with thymus-dependent antigen. Our results demonstrate that *in vivo* OBF-1 is not required for initial

transcription of immunoglobulin genes or for B cell development, but instead is essential for the response of B cells to antigens, and is required for the formation of germinal centres.

The variable region promoters of immunoglobulin genes are selectively activated in B lymphocytes⁴. Based on studies involving *in vitro* transcription⁵, cell transfection^{6–8} and transgenic mice⁹, it seems that the main regulatory element mediating the B-cell-specific activity immunoglobulin promoters is the highly conserved octamer motif ATGCAAAT⁴. B cells contain two POU domain transcription factors that interact specifically with the octamer site: Oct-1, a ubiquitous protein¹⁰, and Oct-2, a largely B-cell-restricted protein^{11–14}. It was initially thought that the high activity of the octamer site in B cells was mediated by the B-cell-restricted Oct-2 protein. However, targeted disruption of the *Oct-2* gene in mice or in a B-cell line showed that Oct-2 is not required for immunoglobulin gene transcription^{15–17}. This seeming paradox was explained by the genetic^{17,18} and biochemical¹⁹ identification of B-cell-restricted transcriptional coactivators for Oct factors. Such a coactivator has been cloned and called OBF-1 (ref. 1), Bob-1 (ref. 2) or OCA-B (ref. 3). OBF-1 is a novel protein expressed throughout B-cell differentiation²⁰, which can be recruited to octamer-containing promoters through interaction with the POU domain of Oct-1 or Oct-2 (ref. 1).

It has been widely assumed that OBF-1 is the crucial factor for immunoglobulin promoter activity in B cells^{1–3,19}. To test this hypothesis we inactivated the *OBF-1* gene. A targeting vector was prepared that replaces most of the coding region by a pGK-neomycin expression cassette (Fig. 1a). Two targeted embryonic stem cell clones (Fig. 1b) were used to generate mice that gave germline transmission of the mutated allele. After crossing with C57BL/6 mice, the resulting heterozygous animals were interbred, and *OBF-1*^{-/-} homozygous mice were obtained at the expected frequency (Fig. 1c) and appeared healthy and vital. Complete inactivation of the *OBF-1* gene was confirmed by northern blot analysis of spleen RNA (Fig. 1d).

Because OBF-1 is already expressed by the early stages of B-cell development and has been implicated in the control of immunoglobulin gene transcription, we reasoned that the absence of this coactivator would affect B-cell differentiation through impair-

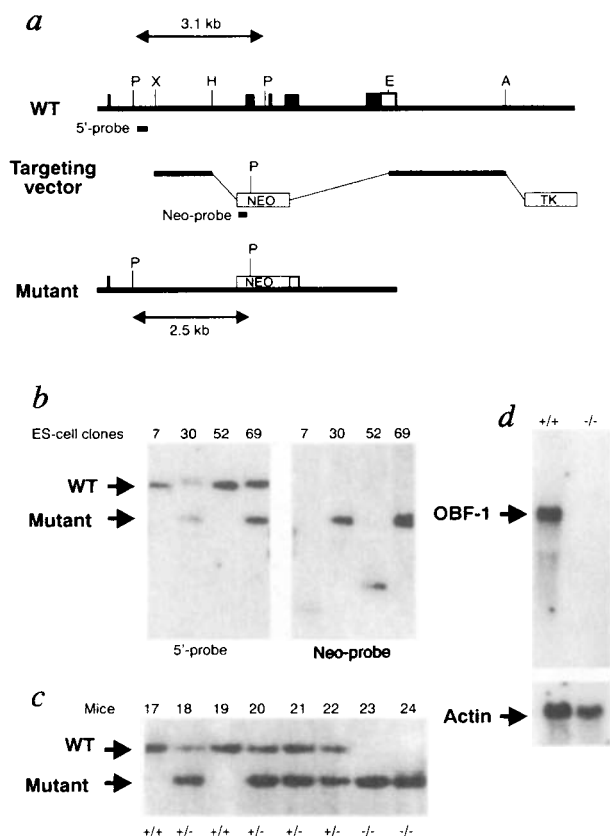


FIG. 1 Generation of OBF-1-deficient mice. *a*, The wild-type allele (WT), the targeting vector and the mutant allele are shown. Black boxes represent the coding exons, the white box symbolizes the 3'-untranslated region in exon 5. Restriction enzyme sites used for cloning and Southern blot analysis are indicated (A, Asp718; E, EcoRV; H, HindIII; P, PstI; X, XbaI). The double-headed arrows illustrate the fragment sizes of the wild-type and mutant alleles when Southern blot analysis is performed with PstI-digested genomic DNA hybridized to the indicated 5'-probe located outside of the area of homologous recombination. Insertion of the targeting vector replaces 250 of 256 amino acids of the OBF-1 protein with the pGK-Neo cassette. *b*, Southern blot analysis of genomic DNA prepared from neomycin- and gancyclovir-resistant embryonic stem (ES) cell clones hybridized to the 5'-probe and to the Neo-probe indicated in *a*. Of 297 double-resistant ES cell clones, two (numbers 30 and 69) were found to have targeted the OBF-1 gene. Both targeted ES cell clones were used for the generation of chimaeric mice, and the resultant mice showed no difference during the analysis. *c*, Southern blot analysis of PstI-digested mouse tail DNA obtained from the offspring of a cross between heterozygous animals. The genotype of each mouse is shown below the corresponding lane. *d*, Northern blot analysis of total spleen RNA obtained from 4-week-old wild-type and mutant littermates, hybridized to a labelled mouse OBF-1 cDNA probe and subsequently to a mouse actin probe (bottom).

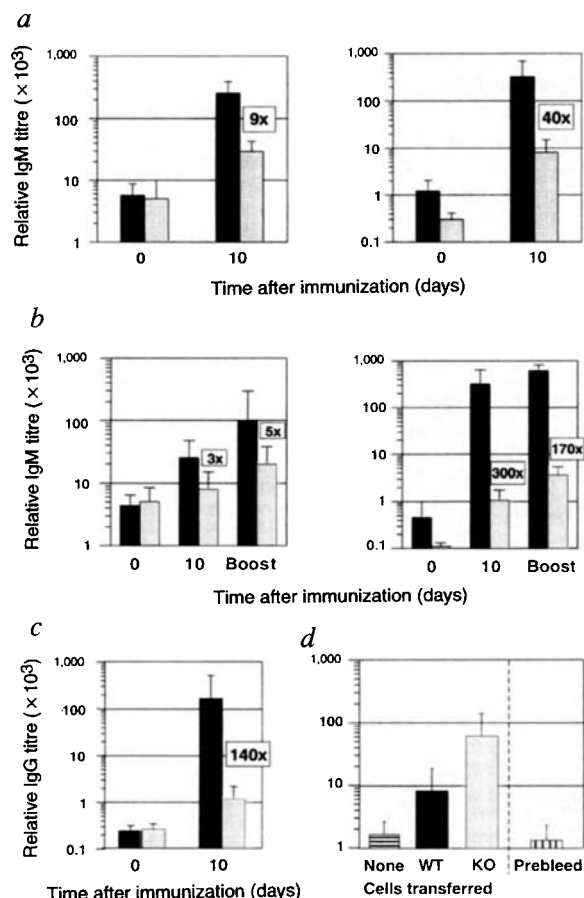
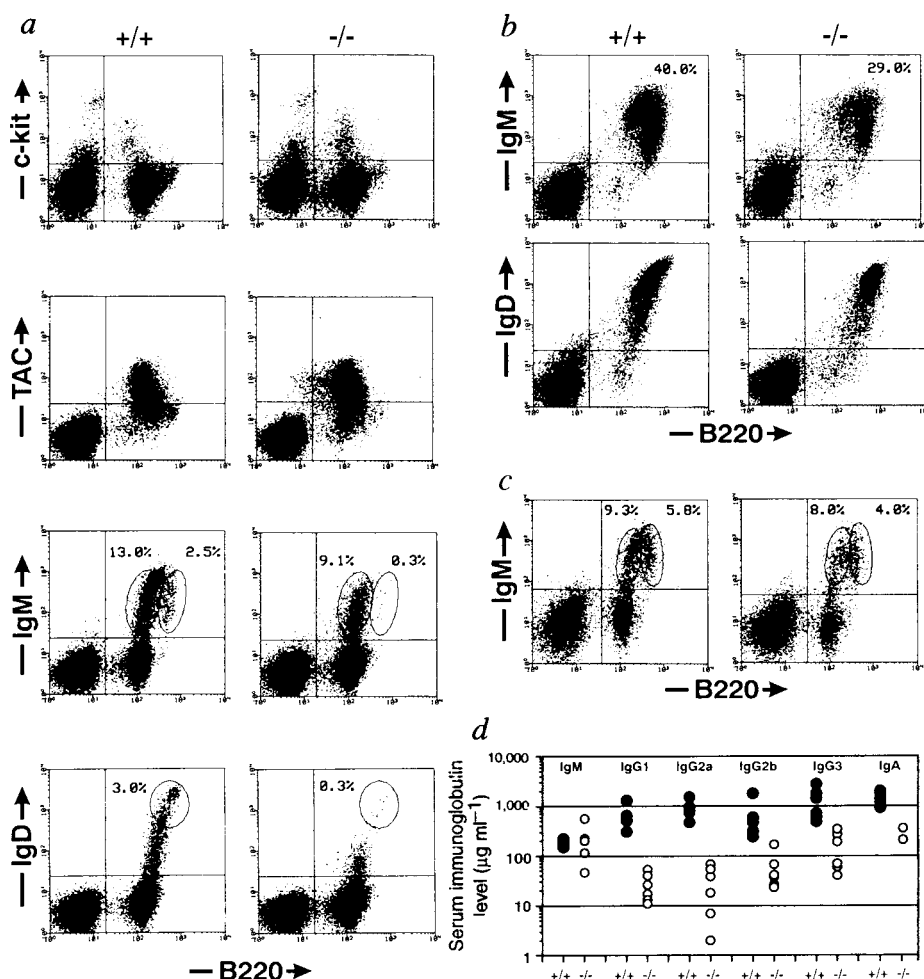
ment of immunoglobulin gene transcription. No difference was observed in B-cell numbers in the bone marrow, but in mutant mice a 2–4-fold reduction in the number of splenic B cells was detected; the spleens had a moderately reduced size and were filled with cells of lymphoid origin (data not shown). Flow cytometric analysis of bone marrow and spleen cells was performed with B-cell stage-specific monoclonal antibodies^{21,22} (Fig. 2). A clear difference between mutant and control mice was observed when cells were stained for B220 and IgM or IgD, respectively. Mature, recirculating B-cells (B220^{high}/IgM^{low} and coexpressing surface IgD) were markedly reduced in bone marrow of four-week-old mutant mice (Fig. 2a); in older mice, some of these cells reappeared (Fig. 2c). In the spleen of OBF-1-deficient mice, mature B-cells were also present at reduced levels (Fig. 2b). Cells representing earlier stages of B-cell differentiation

were unaffected by the OBF-1 mutation, and were present in normal numbers (Fig. 2a). Analysis of additional marker proteins such as interleukin-7 receptor, CD19, CD21, CD43 and CD40 further indicated that early B-cell development occurred normally in the absence of OBF-1 (data not shown). In addition, Mac1 positive myeloid cells and T cells, as analysed by CD4-, CD8- and CD3-specific antibodies, were unaffected by the lack of OBF-1, although the relative numbers of these lymphocytes were moderately increased in the spleen (data not shown).

To investigate whether the OBF-1 mutation had an impact on immunoglobulin production we determined the levels of secreted serum immunoglobulins using an enzyme-linked immunosorbent assay (ELISA). OBF-1^{-/-} mice had greatly reduced levels of all IgG subclasses and IgA (Fig. 2d), indicating a potential isotype switching defect *in vivo*. In contrast, the concentration of IgM in the serum of mutant mice was unaffected, further indicating that immunoglobulin transcription proceeded normally in these mice. In accord with this finding, RNA analysis from spleen or B-cell cultures from normal and mutant mice did not show any difference in the levels of μ or κ immunoglobulin mRNA (data not shown). From these data we conclude that OBF-1 has a strong influence on the production of most immunoglobulin isotypes, but not IgM.

The reduced levels of serum IgGs and IgA in unchallenged OBF-1-deficient mice prompted us to investigate the humoral immune response of these animals. We immunized OBF-1-deficient and normal littermates with NIP-Ficoll, a thymus-independent antigen, or with NIP-ovalbumin (NIP-Ova), a thymus-dependent antigen, and measured the resulting antibody titres with an ELISA that detects NIP-specific antibodies. For the thymus-independent antigen, preimmune sera had indistinguishable titres of IgM anti-NIP-specific antibodies (Fig. 3a). Ten days after immunization, the IgM response of OBF-1-deficient mice was reduced about 10-fold, and the IgG response was reduced about 40-fold. In the case of the thymus-dependent antigen NIP-Ova, lack of OBF-1 affected the IgM response only weakly, but led to a dramatic 300-fold lower production of anti-NIP-specific IgGs by B cells (Fig. 3b). After a secondary immunization eight weeks later, the recall response was only marginally better, and mutant mice still had a drastically reduced titre of specific IgGs. Thus OBF-1 seems to be essential in the immune responses to both thymus-independent and thymus-dependent antigens. To test whether the observed *in vivo* defects might be due to an intrinsic incapacity of mutant B cells to proliferate or secrete Ig after antigen stimulation, several *in vitro* experiments were performed. Splenic B cells were stimulated with various agents including lipopolysaccharide, anti-CD40 monoclonal antibodies (each time alone or in combination with interleukin-4) or anti-IgM, and cellular proliferation and immunoglobulin production were measured. Surprisingly, in all cases the responses were either normal or close to normal. Furthermore, the costimulatory molecule B7.2 was also properly upregulated when splenic cells were stimulated with lipopolysaccharide or anti-CD40 monoclonal antibodies (data not shown). These observations led us to consider the possibility that OBF-1-deficient mice might have an architectural defect in lymphoid organs which would preclude the appearance of a normal immune response. Many of the events that are relevant for normal immunological responses, such as B-cell clonal expansion, somatic hypermutation and isotype switching, all take place in the germinal centres^{23,24}. Formation of germinal centres occurs inside B-cell follicles when stimulated with T-cell-dependent antigens, and requires the coordinated interplay of follicular dendritic cells (FDCs), T cells and B cells²⁴. Because the T-cell-dependent antigen response was nearly absent in OBF-1-deficient mice, we examined whether formation of germinal centres was affected in mice immunized with NIP-Ova. Cryosections of spleens from immunized wild-type and OBF-1 mutant mice were prepared and labelled with antibodies directed against molecules expressed by B cells, T cells or FDCs. Serial sections immunohistochemically double labelled for

FIG. 2 B-cell development in *OBF-1*-deficient mice. Representative flow cytometric analysis of bone marrow (a) and spleen cells (b) from 4-week-old wild-type and *OBF-1*-deficient mice. c, Representative flow cytometric analysis of bone-marrow cells from 20-week-old wild-type and mutant littermates. The $\text{IgM}^{\text{low}}/\text{B220}^{\text{high}}$ and the $\text{IgM}^{\text{high}}/\text{B220}^{\text{high}}$ cells are framed and their percentage is denoted. d, Serum antibody levels in *OBF-1*-deficient mice. Wild-type (filled circles) and mutant mice (open circles; 6 animals of each genotype) 10 weeks old were analysed by sandwich ELISA, or by radial immunodiffusion (IgA determination). The immunoglobulin subclass is indicated at the top of each compartment. In the case of IgA, four of the *OBF-1*^{-/-} mice had titres below the detection limit of the assay ($200 \mu\text{g ml}^{-1}$).



either Thy-1 (green, Fig. 4a, b) or major histocompatibility complex (MHC) class II (green, Fig. 4c, d) and IgM (red, Fig. 4a) showed normal development of primary B-cell follicles (arrows) and T-cell zones in mutant mice. However, in sharp contrast to the wild-type (arrowheads in Fig. 4a, c), mutant mice (Fig. 4b, d) completely lacked germinal-centre microenvironments. This was confirmed by labelling with peanut agglutinin (PNA), a lectin that binds to germinal-centre B cells; this was completely negative in the mutant mice (compare Fig. 4f with Fig. 4e, the wild type). In addition, the presence of immune complex trapping FDCs could be clearly identified in the wild-type mice (Fig. 4g) using the anti-FDC monoclonal antibody FDC-M1 (green) and anti-IgM (red; green and red results in the bright yellow labelling of the FDCs). In contrast, the mutant mice had minimal FDC development (arrows in Fig. 4b, d, f). To determine whether the defect was intrinsic to B cells, additional experiments were performed. Bone marrow from

FIG. 3 Severely impaired immune responses in mice lacking *OBF-1*. a, Relative anti-NIP-IgM (left) and anti-NIP-IgG (right) antibody titres in mice immunized with the thymus-independent antigen NIP-Ficoll. b, Relative anti-NIP-IgM (left) and anti-NIP-IgG (right) antibody titres in mice immunized with the thymus-dependent antigen NIP-ovalbumin. c, Relative anti-NIP-IgG antibody titres in radiation chimaeras reconstituted with wild-type or *OBF-1*^{-/-} bone marrow and subsequently immunized with NIP-Ova. d, Relative anti-NIP-IgG antibody titres in nude mice immunized with NIP-ovalbumin, after transfer of wild-type (WT) or *OBF-1*^{-/-} (KO) CD4⁺ T cells. The origin of the T cells transferred is indicated below the graph. Black bars represent control animals, grey bars represent *OBF-1*-deficient mice. Data are shown as averages ($\pm$ s.d.). The framed numbers show the relative reduction of the antibody titers between control and *OBF-1*-deficient mice.

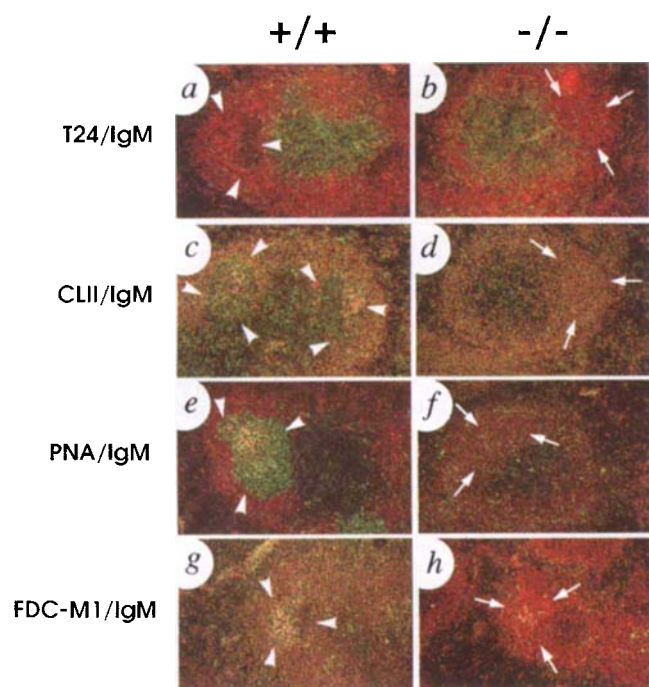


FIG. 4 Lack of FDC development and germinal centre formation in spleens of *OBF-1*^{-/-} mice. *a, c, e, g*, Wild-type mice; *b, d, f, h*, *OBF-1*-deficient mice. The areas within the arrowheads represent germinal centres; the arrows indicate primary follicles only. *a* and *b*, Note the presence of comparable T-cell areas (thy-1, green). *c, d*, Note the presence of both MHC class II positive (green) interdigitating cells in the T-cell areas and MHC class II (green)/IgM (red) double-positive (yellow) follicular B cells. *g, h*, Note the extent of FDC development, as indicated by the bright yellow colour generated by labelling for both the FDC-M1 antigen (green) and immune complexes containing IgM (red). *e, f*, Additional evidence for the ability to form germinal centres using the lectin PNA, which labels germinal centre cells. As can be observed by evaluating all sections, B cells in the *OBF-1*^{-/-} mice (IgM, red) form only primary follicles (arrows) and not germinal centres (arrowheads), as the wild-type mice do in response to an antigenic stimulus.

mutant or normal animals was adoptively transferred into lethally irradiated wild-type littermates which, after reconstitution, were immunized. Mice reconstituted with *OBF-1*^{-/-} bone marrow had a dramatically reduced immune response (Fig. 3c), which indicates that the defect was transferred and therefore does not lie with the FDCs²⁵. Although *OBF-1* is not known to be expressed in T cells, T-cell function was also tested. For this, nude mice were injected with *OBF-1*^{-/-} or wild-type CD4⁺ T cells and subsequently immunized. Transfer of *OBF-1*^{-/-} T cells allowed efficient production of antigen-specific IgGs (Fig. 3d), indicating that T-cell function is normal. In another experiment, lymph-node T cells of either genotype were primed *in vivo* and their capacity to proliferate was measured after antigenic restimulation *in vitro*; once again, *OBF-1*^{-/-} T cells were found to be largely normal (not shown).

Our results show that elimination of the *OBF-1* coactivator does not affect the initial transcription of immunoglobulin genes or early B-cell development. This result, surprising in view of other experiments^{1-3,17,18}, indicates that, with respect to immunoglobulin promoter activation, *OBF-1* and Oct-2 might be functionally redundant. Yet the severely impaired immune response observed in *OBF-1*-deficient animals suggests a unique function for this factor in B cells, that probably lies outside the control of immunoglobulin gene transcription. Presumably, gene(s) critical for efficient immune response and formation of germinal centres appear to require *OBF-1* for their expression. In mice deficient in CD40 (ref. 26), CD40 ligand (ref. 27) or the tyrosine kinase lyn (ref. 28), germinal centres are also affected. However, because the phenotypes of these mice are different from that of *OBF-1*-

deficient mice, it is likely that *OBF-1* controls component(s) of a novel pathway leading to formation of germinal centres.

Note added in proof: Similar results are also described by Kim *et al.* on pages 542–547 of this issue. □

Methods

Plasmid construction. For the construction of the targeting vector a 1.3-kb *Xba*I–*Hind*III fragment from intron 1 isolated from a genomic subclone was cloned upstream of a pGK promoter-driven neomycin expression cassette to give pShort-Neo. Subsequent cloning of an 8-kb genomic *Eco*RV–*Asp*718 fragment, containing part of the untranslated segment of exon 5, into pShort-Neo between the neomycin and the cytomegalovirus promoter-driven thymidine kinase gene gave the targeting vector. Embryonic stem cells (line E14) were propagated, electroporated and selected²⁹. Preparation of genomic DNA, Southern blot and northern blot analyses were performed according to standard procedures. Chimaeric mice were generated by aggregation³⁰, and all mice were maintained in a conventional animal facility.

Flow cytometry. Single-cell suspensions of lymphoid tissues were prepared, stained with the FITC-labelled monoclonal antibody RA3.6B2 (anti-CD45R/B220; Pharmingen) and the PE-conjugated monoclonal antibodies ACK4 (anti-kit), 7D4 (anti-CD25/TAC; Pharmingen), M41 (anti-IgM) and NIM-R9 (anti-IgD) according to standard procedures³² and analysed on a FACScan (Beckton and Dickinson) by gating on living cells based on propidium iodide exclusion (25,000–50,000 events were counted per dot plot).

Serum immunoglobulin detection. Serum immunoglobulins were measured by sandwich ELISA using unlabelled anti-mouse immunoglobulin subclass-specific antibodies as capture reagents and alkaline phosphatase-labelled anti-mouse immunoglobulin-subclass-specific antibodies as developing reagents (Southern Biotechnology Associates). For IgA determination, an IgA Nanorid kit was used (The Binding Site, Birmingham, UK). Anti-NIP antibody titres were measured by ELISA using BSA-coupled NIP as capture reagents and alkaline phosphatase-labelled anti-mouse-IgM or anti-mouse-IgG antibodies (Southern Biotechnology Associates) as developing reagents. The titre was defined as the serum dilution at which the absorbance at 405 nm corresponded to the average background level (with PBS alone) plus three standard deviations.

Immunizations. Wild-type/heterozygous and *OBF-1*-deficient mice (6 mice of each genotype) that were 12 weeks old were immunized with 100 µg thymus-independent antigen NIP-Ficoll in PBS (intravenously injected) or with 100 µg thymus-dependent antigen NIP-Ova in complete Freund's adjuvant (subcutaneously injected). Blood samples were taken shortly before immunization and 7 and 10 days after immunization, as well as 7 days after a second immunization boost (with 10 µg NIP-Ova intravenously injected 4–8 weeks after the primary immunization) in the case of the thymus-dependent antigen. For bone-marrow transfers, mice (6 each) were lethally irradiated and injected with 5 × 10⁶ wild-type or *OBF-1*^{-/-} bone-marrow cells. Eight weeks after reconstitution, chimaeras were immunized with NIP-Ova as above, and 10 days later antibody titres were determined by ELISA. To assess the functionality of T cells, nude mice (5 each) were injected with 3 × 10⁶ CD4⁺ T cells obtained by positive selection from lymph nodes of wild-type or *OBF-1*^{-/-} mice. One day later these mice were immunized subcutaneously with NIP-Ova as above, and the resulting antigen-specific IgG titres were determined by ELISA 10 days later. The prebleed was taken shortly before immunization from 5 randomly chosen nude mice from the experiment.

Immunocytochemistry. Cryosections of spleen from control and *OBF-1*-deficient mice were prepared 10 days after secondary immunization with NIP-Ova, a peak time for germinal-centre formation. The expression of cell-surface proteins was visualized using the following reagents: T24 (rat anti-mouse Thy 1), M5/114 (rat anti-mouse MHC class II), and FDC-M1 (rat anti-mouse FDC) revealed by F(ab)2 fragments of mouse anti-rat IgG (H&L) chain-specific FITC (Jackson ImmunoResearch Labs), or biotinylated peanut agglutinin (Vector) revealed using streptavidin-FITC (Sigma) followed by sheep anti-IgM-texas red (Southern Biotechnology Associates).

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CORRESPONDENCE and requests for materials should be addressed to P.M. (e-mail: matthias@fmi.ch).

The B-cell-specific transcription coactivator OCA-B/OBF-1/Bob-1 is essential for normal production of immunoglobulin isotypes

Unkyu Kim*, Xiao-Feng Qin†, Shiaoqing Gong†, Sean Stevens*, Yan Luo*, Michel Nussenzweig† & Robert G. Roeder*

* Laboratory of Biochemistry and Molecular Biology, and † Laboratory of Molecular Immunology, Howard Hughes Medical Institute, The Rockefeller University, New York, New York 10021, USA

OCA-B was initially identified as a B-cell-restricted coactivator that functions with octamer binding transcription factors (Oct-1 and Oct-2) to mediate efficient cell type-specific transcription of immunoglobulin promoters *in vitro*^{1–3}. Subsequent cloning studies led to identification of the coactivator as a single polypeptide, designated either as OCA-B (ref. 3), OBF-1 (ref. 4) or Bob-1 (ref. 5). OCA-B itself does not bind to DNA directly, but interacts with either Oct-1 or Oct-2 to potentiate transcriptional activation^{1–5}. To determine the biological role of OCA-B, we generated OCA-B-deficient mice by gene targeting. Mice lacking OCA-B undergo normal antigen-independent, B-cell differentiation, including appropriate expression of both immunoglobulin genes and other early B-cell-restricted genes. However, antigen-dependent maturation of B cells is greatly affected. The pro-

liferative response to surface IgM crosslinking is impaired, and there is a severe deficiency in the production of secondary immunoglobulin isotypes including IgG1, IgG2a, IgG2b, IgG3, IgA and IgE in OCA-B-deficient B cells. This defect is not due to a failure of the isotype switching process, but rather to reduced levels of transcription from normally switched immunoglobulin heavy-chain loci. In accord with the defective isotype production, germinal centre formation is absent in these mutant mice.

A null mutation in the OCA-B locus was generated by replacing a 4.5-kb genomic fragment encompassing murine OCA-B exons 2, 3 and 4 with a neomycin-resistance cassette (Fig. 1). Two embryonic stem cell clones that had undergone homologous recombination with the targeting vector (Fig. 1a) were injected into blastocysts. Heterozygous offspring of the germline-transmitting chimaeras were interbred to obtain homozygous mice (OCA-B^{-/-}) (Fig. 1b, c) which did not express OCA-B, as determined by northern blot analysis of spleen RNA (Fig. 1d) and immunoblot analysis of bone-marrow-derived Abelson virus-transformed cell lines (not shown). These mice were viable and fertile, and showed no overt physical abnormalities other than generally reduced body weight.

Because OCA-B is required *in vitro* for full activation of immunoglobulin V_H and V_K promoters^{1–3}, we examined immunoglobulin expression *in vivo* in the OCA-B^{-/-} mice. IgM mRNA and protein expression levels, as well as sterile IgM transcripts^{6,7}, were indistinguishable between OCA-B^{-/-} mice and wild-type controls (not shown). Further, as determined by fluorescence-activated cell sorting (FACS), there were normal numbers of B220⁺/CD43⁺ and B220⁺/CD43⁻ precursors in the bone marrow (not shown) and normal numbers of mature surface IgM⁺/IgD⁺ B cells in the spleen (Fig. 2). Thus IgM expression and the differentiation process leading up to the generation of surface IgM-positive mature B cells^{8,9} are unaffected by the lack of OCA-B.

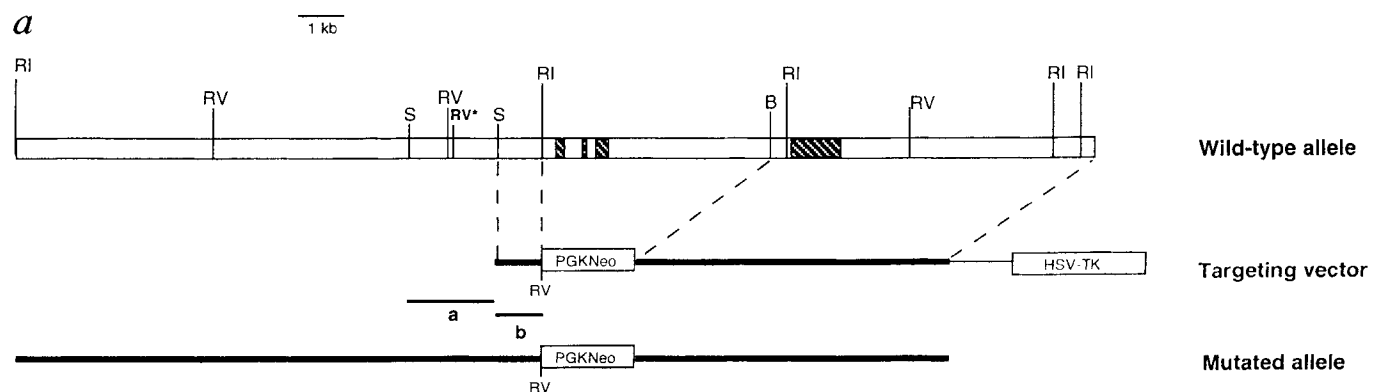
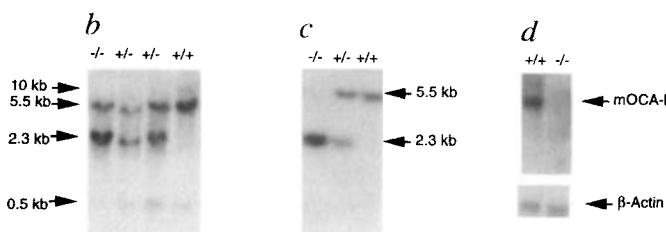


FIG. 1 Targeted inactivation of the OCA-B gene. **a**, Structures of the OCA-B locus, targeting vector and mutant allele. RI, EcoRI; RV, EcoRV; S, SacI; B, BamHI; **b**, **c**, Southern blot analysis of EcoRV-digested genomic DNA of offspring from F₁ heterozygote crossing. Probe **a** detects the wild-type allele as 10, 5.5 and 0.5 kb fragments, and the mutant allele as 5.5 and 2.3 kb fragments. The asterisk indicates an EcoRV polymorphism in the C57BL/6 strain. Probe **b** detects the wild-type allele as a 5.5 kb fragment, and the mutant allele as a 2.3 kb fragment. **d**, Northern blot analysis of OCA-B expression. Poly(A⁺) RNA (1 µg) from spleen is hybridized with OCA-B and β-actin cDNA probes.



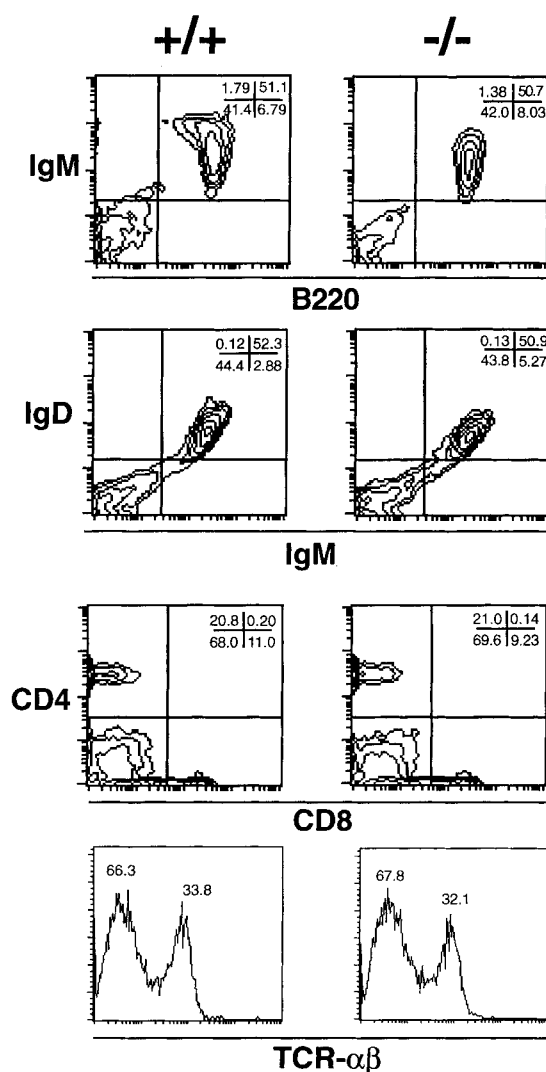


FIG. 2 Representative FACS analysis of splenocytes from wild-type (+/+) and homozygous mutant (-/-) littermates. Mutant mice showed normal B-cell phenotypes in bone marrow and spleen, including B220/I-A, B220/CD23 double staining profiles (not shown).

Furthermore, *OCA-B*^{-/-} mice displayed a normal pattern of CD4/CD8 and T-cell antigen receptor (TCR)-αβ positive T cells (Fig. 2), indicating normal T-cell differentiation in the absence of OCA-B. These results show that the coactivator function of OCA-B is not essential for transcription of immunoglobulin genes, either in the germline configurations or after VDJ recombination in precursor and mature B cells. Our results suggest either that there is a redundant Oct coactivator(s) that can compensate for loss of OCA-B function, or that an OCA-B-like coactivator function is not required in early B-cell differentiation.

We next examined OCA-B function in later stages of B-cell differentiation. Late B-cell maturation is characterized by rapid proliferation upon antigen stimulation, followed by isotype switching and terminal differentiation into antibody-secreting plasma or memory cells¹⁰⁻¹². To determine the B-cell activation potential of OCA-B-deficient mice, proliferative responses to polyclonal mitogens and cytokines were measured¹⁰. Splenic B cells from *OCA-B*^{-/-} mice proliferated at a slightly reduced (~70% of the wild-type) level when stimulated with lipopolysaccharide (LPS) (Fig. 3). However, the proliferative response to stimulation by anti-IgM (anti-μ) crosslinking was approximately fourfold lower for *OCA-B*^{-/-} B cells. The response returned to 90% of the wild-type level by addition of interleukin (IL)-4, suggesting that *OCA-B*^{-/-} B cells can respond to activation signals

generated by T cells. Furthermore, FACS analyses showed that expression of activated B-cell surface markers, such as major histocompatibility complex class II (I-A) and CD23, which are known to be upregulated by LPS and IL-4 treatment¹³, were normal in *OCA-B*^{-/-} mice (data not shown). These data indicate that activation pathways induced by LPS and IL-4 are largely intact in *OCA-B*^{-/-} B cells. In addition, *OCA-B*^{-/-} splenocytes proliferated at a normal level when stimulated by the T-cell mitogen concanavalin A (Fig. 3), suggesting normal function of T cells in *OCA-B*^{-/-} mice. Thus OCA-B function may be required for regulation of specific factors involved in surface IgM-induced signal transduction leading to B-cell proliferation.

To assess the role of OCA-B in production of immunoglobulin isotypes, we measured serum immunoglobulin levels by using an enzyme-linked immunosorbent assay (ELISA) (Fig. 4). The amount of serum IgM was generally higher in *OCA-B*^{-/-} mice than in wild-type mice. Conversely, the levels of secondary isotypes, including IgG1, IgG2a, IgG2b, IgG3, IgA and IgE were markedly reduced in *OCA-B*^{-/-} mice. This indicates that *OCA-B*^{-/-} mice inefficiently produce switched immunoglobulin isotypes. Antigen-dependent maturation of B cells, including isotype switching, takes place largely within the germinal centres of the secondary lymphoid organs such as the spleen and lymph nodes¹⁴. In accord with the diminished isotype production in *OCA-B*^{-/-} mice, there was a striking absence of germinal centres in the lymph nodes of *OCA-B*^{-/-} mice, whereas wild-type and heterozygote littermates contained many well-defined germinal centres (Fig. 5).

To investigate whether reduced isotype production is due to a defect in the switching process, we used FACS to determine the number of splenic cells expressing secondary isotypes. In unimmunized mice the numbers of B cells expressing IgG1, IgG2a, IgG2b and IgG3 were indistinguishable between *OCA-B*^{-/-} and wild-type mice (Fig. 6). To study the switch process in a defined system, B cells isolated from spleen were induced to undergo isotype switching in culture by stimulation with LPS and cytokines. It has been shown that splenic B cells stimulated with LPS undergo switching to IgG2b and IgG3, whereas LPS and IL-4 in combination stimulate IgG1 and IgE switching¹¹. The numbers of surface immunoglobulin-expressing cells induced by LPS (IgG2b and IgG3), or by LPS and IL-4 (IgG1 and IgE), were indistinguishable between *OCA-B*^{-/-} and wild-type mice (Fig. 7). Furthermore, enzyme-linked immunospot (ELISPOT) assays showed that the numbers of cells secreting IgG2b and IgG3 (LPS), and IgG1 (LPS + IL-4), were comparable for mutant and wild-type B cells (Table 1). However, when normalized to the number of cells

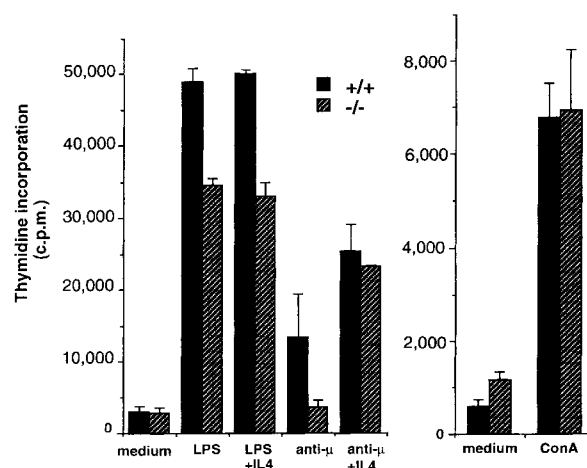


FIG. 3 Proliferative response of splenocytes to polyclonal mitogens and cytokines. T-cell-depleted and Percoll gradient-purified resting B cells were stimulated with the indicated mitogens. For ConA stimulation, red blood- and dead cell-depleted splenocytes were used. Five wild-type and mutant littermates were analysed in three separate experiments, and representative data are shown.

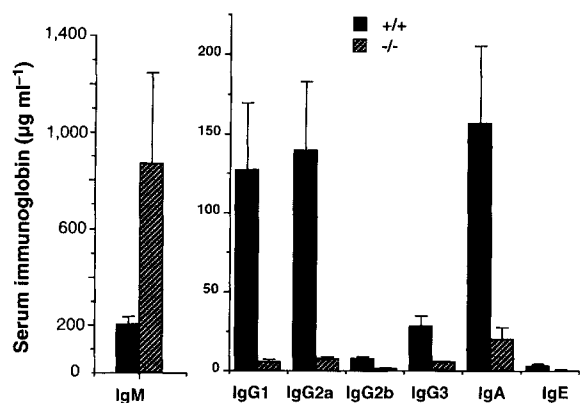


FIG. 4 Serum antibody levels of unimmunized animals. Immunoglobulin isotypes were measured by ELISA. Average titres from five wild-type and *OCA-B*^{-/-} mice (8–20 weeks old) are shown.

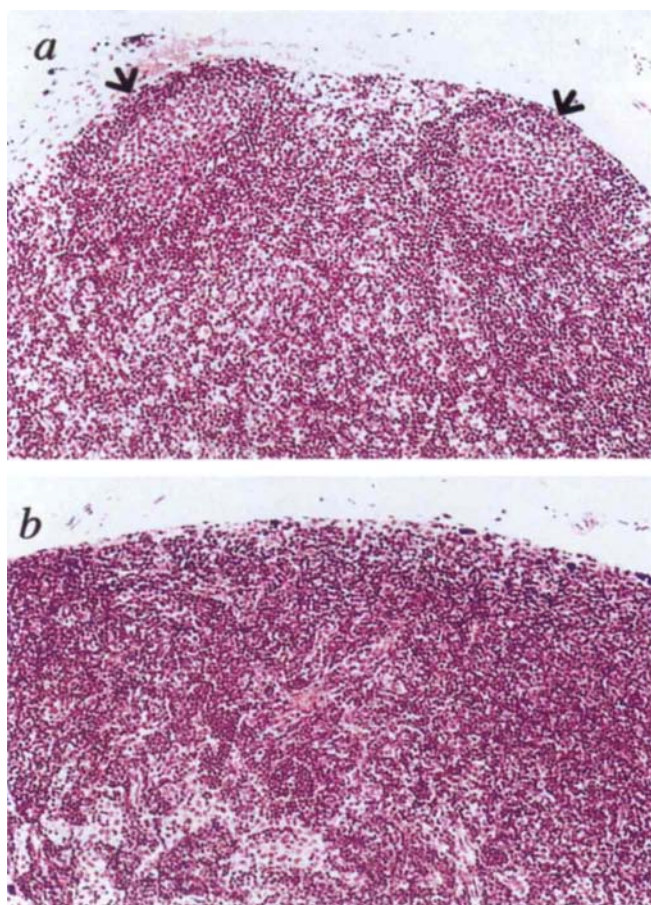


FIG. 5 Histological analysis of mesenteric lymph nodes of 20-week-old wild-type (a) and *OCA-B*^{-/-} (b) littermates. Mesenteric lymph nodes were isolated, and paraffin-embedded sections were stained with eosin and haematoxylin. Arrows indicate germinal centres which are absent in the mutant mice. A total of three homozygous, one heterozygote and two wild-type mice were examined. None of the *OCA-B*^{-/-} mice contained germinal centres in the secondary lymphoid organs, including mesenteric and cervical lymph nodes, Peyer's patches and spleen. Original magnification, $\times 20$.

secreting each isotype, the rates of IgG2b and IgG3 secretion stimulated by LPS and the rate of IgG1 secretion stimulated by LPS with IL-4 were fivefold and tenfold lower, respectively, in *OCA-B*^{-/-} than in wild-type B cells (Table 1). These data suggest that *OCA-B*^{-/-} B cells are intrinsically capable of undergoing the isotype switch processes *per se*, but are inefficient at expressing the switched immunoglobulin genes. In agreement with the demonstration of normal isotype switching in *OCA-B*^{-/-} B cells, northern blot analyses showed that the levels of sterile switch transcripts (which are proposed to be prerequisites for isotype switching) induced by LPS (I γ 2b)¹⁵ or LPS plus IL-4 (I γ 1)¹⁶ were indistinguishable between the mutant and wild-type mice (Fig. 8a). In contrast, although similar amounts of IgM (μ) transcripts were detected, the levels of GAPDH-normalized secondary immunoglobulin isotype transcripts were significantly lower in *OCA-B*^{-/-} B cells in response to LPS (pan- γ and C γ 2b, 3–4-fold reduction) or LPS plus IL-4 (pan- γ and C γ 1, 5–9-fold reduction; IgE(ϵ), twofold reduction) (Fig. 8b, c). Taken together, the results show that *OCA-B* is essential for normal levels of transcription from switched immunoglobulin loci, but not for switching itself.

Cell type-specific transcription of immunoglobulin genes is achieved by interactions between variable-region promoters and multiple enhancer-bound factors¹⁷. Our results demonstrate a differential requirement for *OCA-B* during certain stages of B-cell differentiation. Although light and heavy μ chain expression during antigen-independent B-cell differentiation does not require *OCA-B*, normal expression of immunoglobulin genes in

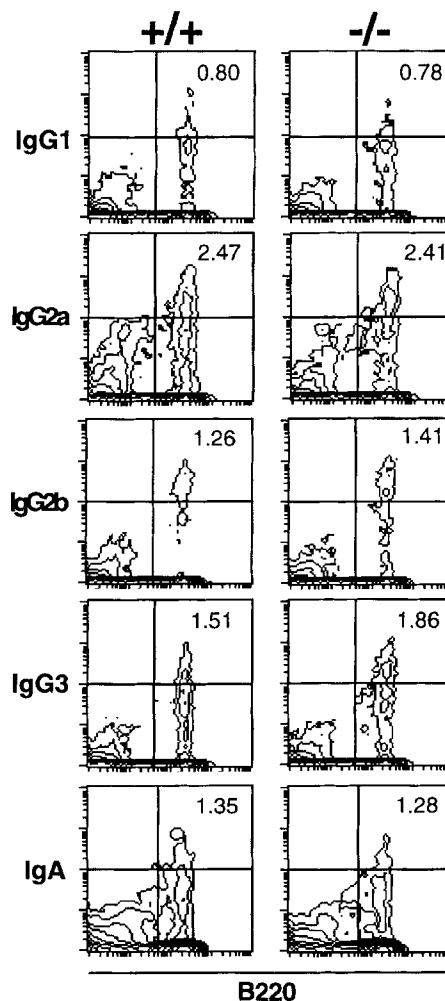


FIG. 6 Surface expression of immunoglobulin isotypes on mature splenic B cells. Double staining with B220 and isotype-specific antibodies was used to identify surface positive cells in spleens of unimmunized mice. Numbers indicate the percentage of isotype-positive cells.

TABLE 1 Directed isotype switching in culture

(a) Number of cells secreting immunoglobulins (per 10,000 cells)									
Activation	IgM		IgG1		IgG2b		IgG3		
	+/+	-/-	+/+	-/-	+/+	-/-	+/+	-/-	
None		10							
LPS	2,605	4,155	10	10	164	180	279	225	
LPS + IL-4	482	550	70	181	35	5	15		
(b) Immunoglobulin titre of culture supernatant (pg per positive cell)									
Activation	IgM		IgG1		IgG2b		IgG3		
	+/+	-/-	+/+	-/-	+/+	-/-	+/+	-/-	
None									
LPS	120	210	13.6	12.9	1.9	0.4	82	18	
LPS + IL-4	932	389	2,479	206					

Rates of immunoglobulin secretion in stimulated cells. The numbers of cells producing each isotype were determined by ELISPOT assay²⁸. The concentrations of immunoglobulin isotypes in the culture supernatants were determined by ELISA. Where no value is given the isotypes were below the level of detection. Mean data obtained from four wild-type and mutant littermates analysed in three separate experiments are shown.

the antigen-dependent phase of B-cell differentiation shows a strict requirement for OCA-B. Furthermore, *in vitro* switching experiments show that although OCA-B is not essential for immunoglobulin heavy-chain switching *per se*, it is essential for normal transcription of the switched immunoglobulin loci. This may be due to alterations in promoter responsiveness to immunoglobulin enhancers by the architectural changes introduced by the switch recombination. Thus the possible loss of other regulatory elements, and/or closer apposition to the promoter of the more distal 3' enhancer implicated in immunoglobulin expression in activated B cells^{18,19}, may increase the dependency of the promoter on OCA-B activity. Alternatively, the levels of factors that are normally used (or able to compensate for OCA-B loss) during early B-cell development may decrease during maturation^{18,20}, thus increasing the reliance upon OCA-B-dependent activation. The ability of OCA-B to enhance 'isolated' V_H (and V_κ) promoters both *in vitro*^{2,3} and in transfected cells^{4,5} is most

consistent with an *in vivo* function at octamer-containing promoters, although an *in vivo* function at enhancer element(s)¹⁷ is not excluded.

OCA-B also may function at other levels in the generation of immunoglobulin isotypes; for example, in the expression of genes encoding regulatory proteins involved either in transcription of the switched immunoglobulin genes or in the cell-cell interactions that are a prerequisite for B-cell activation and terminal differentiation^{12,14}. Preliminary data indicate that antibody responses to T-cell-dependent and T-cell-independent antigens (trinitrophenol (TNP)-LPS and sheep red blood cells (SRBCs)) are reduced in *OCA-B*^{-/-} mice (data not shown). Specifically, although levels of anti-TNP IgM were equal or slightly higher in mutant mice, levels of anti-TNP IgG3 and anti-SRBC IgM and IgG were severely reduced. This suggests that *OCA-B*^{-/-} mice may be inefficient in clearing antigens with secondary isotype antibodies. The persistence of antigens may cause continuous

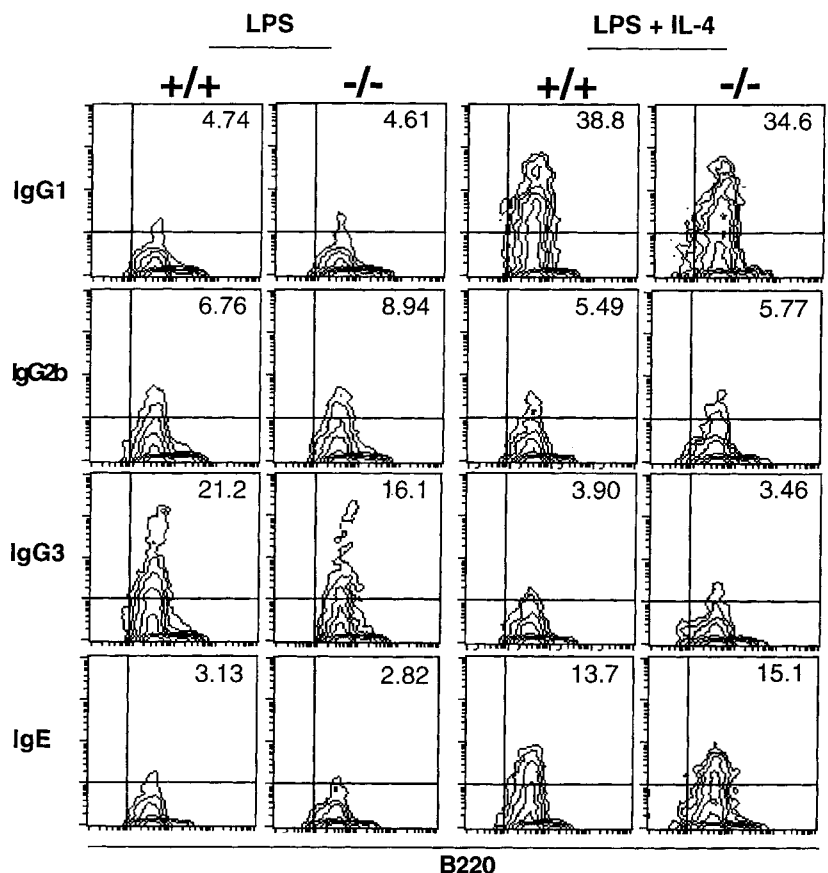


FIG. 7 Surface expression of IgG1, IgG2b, IgG3 and IgE isotypes in stimulated splenic B cells. Cells were analysed after 5 days of stimulation with either LPS or LPS plus IL-4. No difference was seen between +/+ and -/- mice, and representative B220 and isotype-specific double-staining plots of the +/+ and -/- littermates are shown. Positive cells were identified by quadrant gates, and the frequencies (as percentages) are indicated.

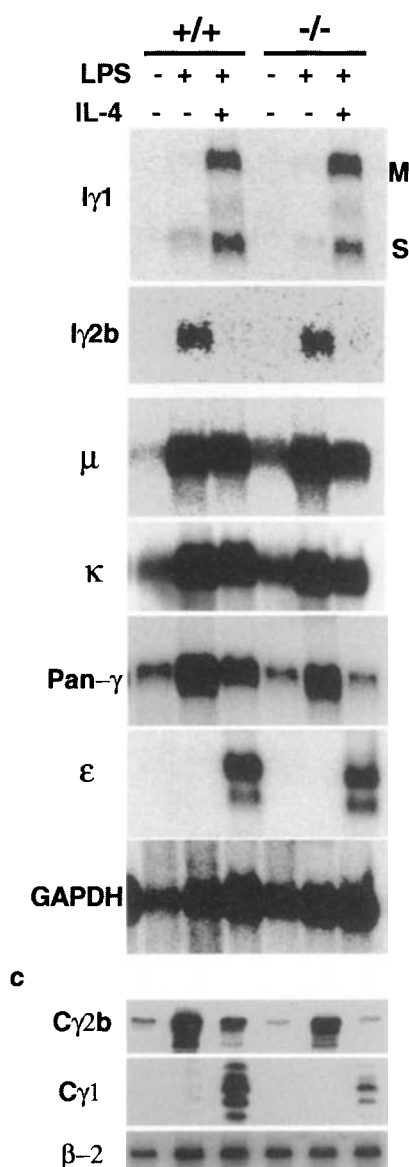


FIG. 8 Expression of germline switch and mature immunoglobulin transcripts in stimulated B cells. *a*, Northern analysis of germline switch transcription. RNAs prepared from untreated, LPS or LPS + IL-4 stimulated splenic B cells were hybridized with Iy1- and Iy2b-specific probes. The Iy1 probe identified both membrane-bound (M) and secreted (S) forms of germline switch transcripts; Iy2b revealed primarily the secreted species. *b*, Northern analysis of mature immunoglobulin mRNA. The same RNA blot used in Fig. 4a was sequentially hybridized with μ , κ , pan- γ (recognizing all four IgG species) and ϵ probes. The ϵ probe detects both the germline switch transcript (1.7–1.9 kb band) and the mature transcript (2.3 kb). *c*, RNase protection analysis for γ 2b (233 bp) and γ 1 (112 bp) RNA.

stimulation of B cells to produce more IgM, leading to elevated levels of serum IgM in *OCA-B*^{-/-} mice (Fig. 4). In agreement with this idea, there were consistently higher numbers of IgM-secreting cells in the mutant mice than in wild-type mice (Table 1). Moreover, the absence of active germinal centre formation in *OCA-B*^{-/-} mice suggests that affinity maturation processes generating high-affinity antibodies might be defective in these mice, further diminishing their ability to remove antigens. Despite uncertainty about the direct target(s) of *OCA-B*, it clearly plays a central role in late B-cell differentiation. Our results also demonstrate that a deficiency in a transcriptional coactivator is manifested as a severe physiological defect, which can serve as a model for studying certain forms of immunodeficiency associated with low levels of secondary immunoglobulin isotypes in humans.

Note added in proof: Similar results are also described by Schubart *et al.* on pages 538–542 of this issue. □

Methods

Targeted disruption of the *OCA-B* gene. A murine *OCA-B* genomic clone was isolated from a 129/Sv genomic library, and the exon/intron structure was determined by restriction enzyme mapping, sequencing and RNase protection analyses. The targeting vector was constructed by replacing a 4.5-kb fragment containing exons 2, 3 and 4 with the neo-resistance cassette in pPNT²¹. The herpes simplex virus thymidine kinase (HSV-TK) gene was inserted downstream of the long arm. The linearized targeting vector was transfected into embryonic stem (ES) cell lines E14 and CJ-7. G418 and gancyclovir-resistant clones were screened for homologous recombination by polymerase chain reaction (PCR) and Southern blotting. Two correctly targeted ES clones were obtained and injected into C57BL/6 blastocysts. Heterozygous offspring of the germline-transmitting chimaeras were interbred to obtain homozygous mice.

In vitro culture of splenic B cells. Splenocytes of mice 10–20 weeks old were depleted of T cells by anti-Thy1.2 antibody and complement treatment (Sigma)²², followed by overnight incubation to remove adherent cells. Resulting B cells were cultured at 5×10^5 cells ml⁻¹ in RPMI medium supplemented with 10% fetal calf serum, 2 mM L-glutamine and 50 μ M 2-mercaptoethanol. LPS was used at 25 μ g ml⁻¹, and IL-4 at 50 ng ml⁻¹ or 50 U ml⁻¹ (Sigma and Beckton Dickinson) for all *in vitro* stimulation assays. For surface IgM crosslinking assay, 20 μ g ml⁻¹ anti- μ (F(ab')₂) fragment of goat anti-mouse IgM (Pierce) was used. The degree of cell death, as measured by trypan blue exclusion assay, in wild-type and mutant cultures was similar.

FACS analysis. Wild-type (+/+), heterozygous (+/-) and homozygous (-/-) littermates 6–12 weeks of age were used for FACS analysis. Because no difference was observed between +/+ and +/- animals, only the results from +/+ and -/- littermates are shown. The following antibody conjugates were used: fluorescein (FITC) goat anti-mouse IgM (Jackson); phycoerythrin (PE) goat anti-mouse IgD (Southern Biotechnology); PE-anti-mouse B220 (RA36B2, Pharmingen); FITC-anti-mouse TCR- $\alpha\beta$, PE-anti-mouse CD4 and R613 anti-mouse CD8a (H57-597, H129.19 and 53-6.7; GIBCO-BRL). 5×10^3 events were analysed with a live lymphocyte gate as defined by light scatter (FACScan, Becton-Dickinson). For determining cell surface expression of isotypes, fresh splenocytes or stimulated splenic B cells were first incubated with a Fc γ II/III receptor blocker (2.4G2), then stained with different biotinylated isotype-specific polyclonal or monoclonal antibodies and FITC-anti-mouse B220 (RA36B2, Pharmingen). The biotinylated antibodies were revealed with RED-670 streptavidin (GIBCO-BRL). The isotype-specific antibodies used in the experiments were: polyclonal goat anti-mouse IgG2a and IgA (Southern Biotechnology) and monoclonal anti-mouse IgG1, IgG2b, IgG3 and IgE (G1-6.5, R12-3, R40-82 and R35-92; Pharmingen). 20×10^3 events were analysed with a live cell gate as defined by light scatter.

ELISA and ELISPOT assay. Serially diluted sera or 5-day B-cell culture supernatants were added to plates coated with isotype-specific capture antibody (Southern Biotechnology). Bound immunoglobulin was detected by biotinylated isotype-specific antibodies and avidin-peroxidase (Sigma). Concentration was determined using purified immunoglobulins (Sigma) as standard. For ELISPOT assays serially diluted B cells stimulated for 5 days as described above were plated on ELISA plates coated with capture antibodies. Plates were developed after 20 hours using biotinylated antibody and anti-biotin peroxidase conjugate (Sigma). Resulting spots were counted with the aid of a dissecting microscope.

Proliferation assay. T-depleted B cells were further purified by a discontinuous Percoll gradient consisting of 75, 70, 65, 60, 53 and 43% Percoll solution, and spun at 1,900g for 30 min at 4 °C. The high-density cells (60% and 65% Percoll fraction) were recovered, washed extensively, and cultured in triplicate in 96-well plates at 5×10^5 cells ml⁻¹ (100 μ l per well). For concanavalin A (ConA) stimulation, total splenocytes (depleted of red blood cells and dead cells) were cultured with 5 μ g ml⁻¹ ConA (Sigma). After 48–72 h, the cultures were pulsed with 1 μ Ci [³H] methyl-thymidine for 12 h, and incorporation of thymidine was measured.

Northern and RNase protection analysis. Total RNA was prepared from splenic B cells stimulated with LPS or LPS plus IL-4 for 4 days, and from fresh splenic B cells as untreated control. RNA (10 μ g) was used for northern hybridization with the following probes: Iy1, 0.7-kb PstI-KpnI fragment from pY1Bx; Iy2b, 0.5-kb HindIII-XhoI fragment of SKA; pan- γ , 1 kb XhoI-KpnI of 3'C γ 2bXR; ϵ , 3 kb SacI of pE^{15.16.23.24} (gifts from A. Bottaro and F. Alt); GAPDH, 1-kb PstI fragment of pR-GAPDH; μ , CH4 of mouse μ heavy chain; κ , Ck of mouse κ light chain²⁵. For RNase protection, 10 μ g RNA was used and the conditions including β -2 microglobulin probe (as RNA control) were as described²⁶. Iy-C γ 2b probe was described²⁷ (a gift from P. Rothman). Quantification was performed with a phosphorimager and ImageQuant 3.3 software (Molecular Dynamics).

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CORRESPONDENCE and requests for materials should be addressed to R.G.R. (e-mail: roeder@rockefeller.edu).

A role for Pyk2 and Src in linking G-protein-coupled receptors with MAP kinase activation

Ivan Dikic*, George Tokiwa*, Sima Lev†, Sara A. Courtneidge† & Joseph Schlessinger*

* Department of Pharmacology, New York University Medical Center, 550 First Avenue, New York, New York 10016, USA
† SUGEN Inc., 515 Galveston Drive, Redwood City, California 94063, USA

THE mechanisms by which mitogenic G-protein-coupled receptors activate the MAP kinase signalling pathway are poorly understood. Candidate protein tyrosine kinases that link G-protein-coupled receptors with MAP kinase include Src family kinases¹, the epidermal growth factor receptor², Lyn and Syk³. Here we show that lysophosphatidic acid (LPA) and bradykinin induce tyrosine phosphorylation of Pyk2 and complex formation between Pyk2 and activated Src. Moreover, tyrosine phosphorylation of Pyk2 leads to binding of the SH2 domain of Src to tyrosine 402 of Pyk2 and activation of Src. Transient overexpression of a dominant interfering mutant of Pyk2 or the protein tyrosine kinase Csk reduces LPA- or bradykinin-induced activation of MAP kinase. LPA- or bradykinin-induced MAP kinase activation was also inhibited by overexpression of dominant interfering mutants of Grb2 and Sos. We propose that Pyk2 acts with Src to link G_i- and G_q-coupled receptors with Grb2 and Sos to activate the MAP kinase signalling pathway in PC12 cells.

PC12 cells were stimulated with lysophosphatidic acid (LPA) and lysates of stimulated and unstimulated cells were immunoprecipitated with antibodies against Pyk2, then immunoblotted with antibodies against phosphotyrosine. Figure 1a shows that tyrosine phosphorylation of Pyk2 is stimulated in response to LPA or bradykinin. Pretreatment of PC12 cells with pertussis toxin inhibited tyrosine phosphorylation of Pyk2 in response to LPA

stimulation (Fig. 1b). However, bradykinin- or phorbol-12-myristate-13-acetate (PMA)-induced activation of Pyk2 was not affected by pretreatment with pertussis toxin. Removal of extracellular calcium by addition of EGTA to the medium did not affect LPA-induced Pyk2 phosphorylation (Fig. 1b). These results indicate that LPA-induced Pyk2 activation is dependent, at least in part, on a pertussis-toxin-sensitive G_i-dependent pathway in PC12 cells. A similar inhibition of LPA-induced tyrosine phosphorylation of Shc and activation of MAP kinase by pertussis toxin has been described and attributed to a G_i-dependent pathway⁴.

We have previously shown that activation of Pyk2 by elevation of intracellular calcium concentration can lead to activation of the MAP kinase signalling pathway in PC12 cells⁵. In addition, Src-family protein tyrosine kinases are activated in response to stimulation of a variety of G-protein-coupled receptors^{1,3,6–8} and are necessary for linking G_i- and G_q-coupled receptors with MAP kinase activation^{1,3}. The epidermal growth factor (EGF) receptor and erbB2 have also been implicated in MAP kinase activation induced by LPA and other agonists of G-protein-coupled receptors². We have therefore tested whether LPA and bradykinin can activate Src and the EGF receptor in PC12 cells. Stimulation of PC12 cells by LPA or bradykinin leads to an approximately 3–4-fold increase in Src kinase activity (data not shown), whereas we were unable to detect LPA-induced activation of EGF receptor in PC12 cells (data not shown).

As Pyk2 and Src are activated in response to LPA or bradykinin stimulation, we tested whether these two protein tyrosine kinases associated with one another in response to stimulation with LPA or bradykinin. Lysates from stimulated or unstimulated cells were immunoprecipitated with antibodies against Src and immunoblotted with antibodies against Pyk2, Src or anti-pY416^{Src} antibodies,

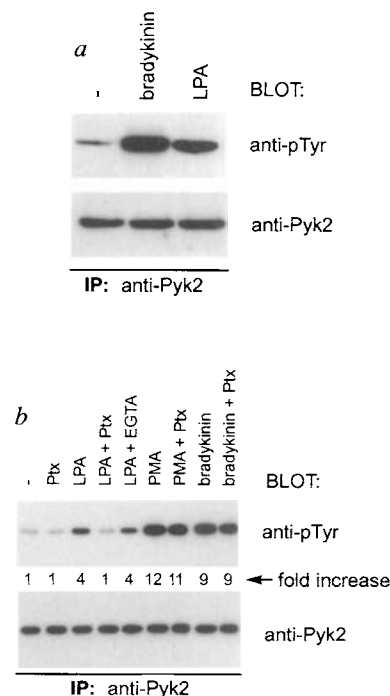
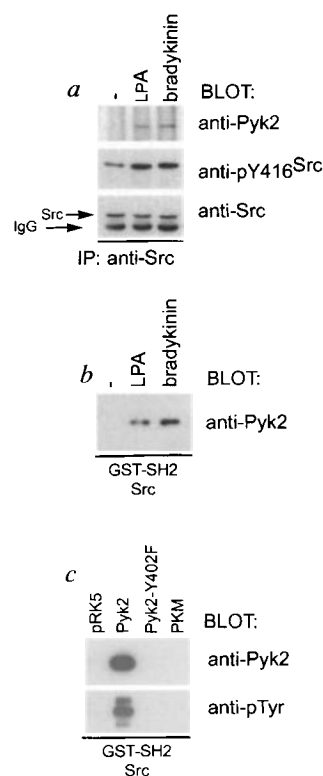


FIG. 1 LPA and bradykinin induce tyrosine phosphorylation of Pyk2 in PC12 cells. **a**, PC12 cells were stimulated with LPA (2.5 μ M) or bradykinin (1 μ M) for 3 minutes at 37 °C and lysed. Pyk2 was immunoprecipitated (IP) with antibodies against Pyk2 and immunoblotted (BLOT) with antibodies against phosphotyrosine (anti-pTyr) or against Pyk2. **b**, PC12 cells were left untreated (–) or incubated with pertussis toxin (Ptx) 100 ng ml^{–1} for 20 h and then stimulated with LPA (2.5 μ M), bradykinin (1 μ M) for 3 min at 37 °C or with PMA (1 μ M) for 10 min at 37 °C. In addition, cells were stimulated with LPA in medium containing 3 mM EGTA. We have analysed phosphorylation of immunoprecipitated Pyk2 by immunoblotting with anti-pTyr or anti-Pyk2 antibodies. The increase in tyrosine phosphorylation of Pyk2 is indicated (fold increase).

FIG. 2 Association between Pyk2 and Src *in vivo* and *in vitro*. **a**, PC12 cells were left untreated (–) or stimulated with LPA (2.5 μ M) or bradykinin (1 μ M) for 3 min at 37 °C. Src was immunoprecipitated (IP) and immunoblotted (BLOT) with antibodies against Src, anti-pY416^{Src} antibodies or antibodies against Pyk2. **b**, The same lysates were mixed with a GST fusion protein containing the SH2 domain of Src and the bound proteins were analysed by immunoblotting with antibodies against Pyk2. **c**, 293T cells were transiently transfected with pRK5 (vector alone), and expression vector containing Pyk2, Pyk2-Y402F or Pyk2 kinase-negative mutant (PKM). The lysates were incubated with a GST fusion protein containing the SH2 domain of Src and bound Pyk2 was analysed by immunoblotting with anti-Pyk2 or anti-pTyr antibodies.



which specifically recognize activated Src⁹. This experiment demonstrated that Pyk2 forms a complex with activated Src in response to LPA or bradykinin stimulation (Fig. 2a). Furthermore, the SH2 domain of Src bound to activated Pyk2 (Fig. 2b), suggesting that Src binding to Pyk2 is mediated by its SH2 domain, perhaps leading to its activation^{9,10}. To analyse further the interaction between Src and Pyk2, we did an *in vitro* binding experiment using a glutathione-S-transferase (GST) fusion protein containing the SH2 domain of Src with wild-type Pyk2, a mutant form of Pyk2 in which tyrosine at position 402 was replaced by a phenylalanine residue (Pyk2-Y402F) or a kinase-negative mutant of Pyk2 (PKM)⁵. A GST fusion protein containing the SH2 domain of Src bound to tyrosine-phosphorylated Pyk2 but not to the Pyk2-Y402F mutant or PKM (Fig. 2c). The tyrosine at position 402 is the principal autophosphorylation site of Pyk2 (I. D. and J. S., unpublished results) and is found within the sequence YAEI, a consensus binding site for the SH2 domain of Src kinases¹¹.

We further examined the status of Src phosphorylation and MAP kinase activation upon transfection of Pyk2 or Pyk2-Y402F in human embryonic kidney 293T cells. Overexpression of Pyk2 leads to ~fourfold stimulation of endogenous Src activity in these cells (Fig. 3a), but overexpression of Pyk2-Y402F or PKM did not affect Src activity in the same assay (Fig. 3a). These results indicate that autophosphorylation of Pyk2 on Tyr 402 leads to binding of the SH2 domain of Src and its subsequent activation. We next overexpressed Pyk2, Pyk2-Y402F or Pyk2 with increasing amounts of Csk, a protein tyrosine kinase that negatively regulates Src¹², and tested whether Pyk2-induced Src activation contributes to Pyk2 tyrosine phosphorylation and Pyk2-induced MAP kinase

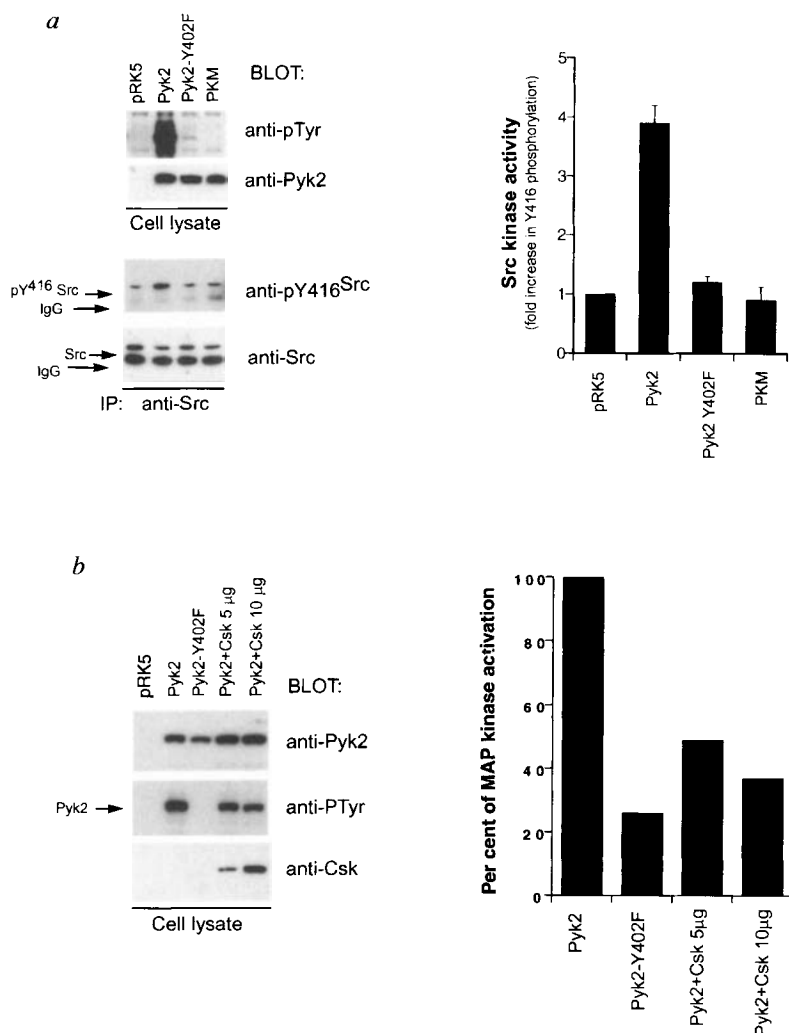


FIG. 3 Pyk2 and Src are involved in MAP kinase activation. **a**, 293T cells were transiently transfected with pRK5, Pyk2, Pyk2-Y402F and PKM. Total-cell lysates were analysed by immunoblotting with anti-pTyr or anti-Pyk2 antibodies (left). The same lysates were subjected to immunoprecipitation with antibodies against Src and immunoblotting with anti-pY416^{Src} or antibodies against Src. Src kinase activity was quantified as an increase in pY416^{Src} phosphorylation and presented as mean $\pm$ s.d. from four independent experiments (right). **b**, The effects of coexpression of Pyk2 and Csk on Pyk2 tyrosine phosphorylation and MAP kinase activation. pRK5, Pyk2, Pyk2-Y402F or Pyk2 plus increasing concentrations of Csk were transiently transfected in 293T cells. Total cell lysates were analysed by immunoblotting with antibodies against phosphotyrosine, Pyk2 and Csk (left). A weak tyrosine phosphorylation of Pyk2 Y402F was observed upon longer exposure (left). The same lysates were used to determine MAP kinase activation. This experiment was repeated three times. The plots show percentage of MAP kinase activity relative to the activity determined for Pyk2 transfected cells (right).

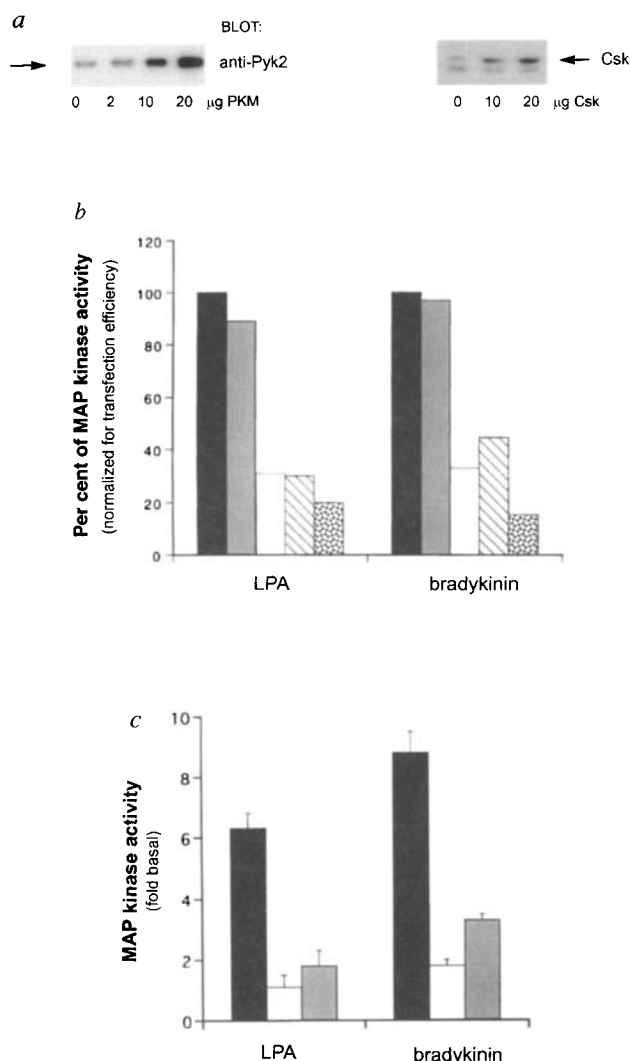


FIG. 4 Effects of dominant interfering mutants on LPA- and bradykinin-induced MAP kinase activation. **a**, PC12 cells were transiently transfected with pRK5, PKM and Csk. Arrow indicates total contribution of endogenous Pyk2 and increasing amounts of PKM recognized by anti-Pyk2 antibodies (left). Expression of Csk was determined by blotting of total cell lysates with anti-Csk antibodies (right). **b**, PC12 cells were mock-transfected (pRK5), or transfected with truncated EGF receptor, PKM, Csk, or with both PKM and Csk, stimulated with LPA or bradykinin for 3 min, lysed and MAP kinase activity determined. The transfection efficiency was determined using a β -galactosidase transgene. Plots show percentage of MAP kinase activity relative to the activity determined for mock-transfected PC12 cells. Similar results were obtained in three independent experiments done in duplicate. Bars represent pRK5 (black), truncated EGF receptor (grey), PKM (white), Csk (hatched) and PKM + Csk (dotted). **c**, Quantification of MAP kinase activity in PC12 cells overexpressing dominant interfering mutants of Grb2 and Sos. Cells were stimulated with LPA or bradykinin and MAP kinase activity determined. Experiments were repeated three times. The plot shows fold increase $\pm$ s.d. of MAP kinase activity over basal level. Bars represent MAP kinase activities in parental PC12 cells (black), PC12 cells expressing a Grb2 deletion mutant lacking the N-terminal SH3 domain (white) and PC12 cells expressing the SH3 binding domain (proline-rich tail) of Sos (grey).

activation in 293T cells. Both tyrosine phosphorylation of Pyk2 and Pyk2-induced MAP kinase activation, normally observed from cells overexpressing Pyk2 (ref. 5), were significantly reduced in the presence of increasing amounts of Csk (Fig. 3b). In addition, MAP kinase activation was greatly reduced in cells overexpressing Pyk2-Y402F compared with MAP kinase activation induced by expression of wild-type Pyk2 (Fig. 3b). Pyk2-Y402F is poorly

tyrosine-phosphorylated upon overexpression (Fig. 3a, b). Moreover, overexpression of Pyk2-Y402F did not activate Src in these experiments (Fig. 3a). These results show that Pyk2 tyrosine phosphorylation and Pyk2-induced MAP kinase activation depend, at least in part, on Src kinase activity stimulated by binding to autophosphorylated Tyr 402 on Pyk2 (Fig. 3).

We next examined the role of Pyk2 in LPA- or bradykinin-induced MAP kinase activation by using the dominant interfering kinase-negative mutant of Pyk2 (refs 5, 13). As we were unable to generate stable PC12 cell lines overexpressing PKM, we used a transient overexpression strategy. Overexpression of PKM in PC12 cells (Fig. 4a, left) strongly inhibited MAP kinase activation by LPA or bradykinin (Fig. 4b). The role of Src in LPA- or bradykinin-induced MAP kinase activation was further tested by transient overexpression of Csk. When Csk was overexpressed in PC12 cells (Fig. 4a, right), a strong reduction in LPA- or bradykinin-induced MAP kinase was observed (Fig. 4b). In cells that were co-transfected with both PKM and Csk, LPA- or bradykinin-induced MAP kinase activation was strongly inhibited (Fig. 4b); however, overexpression of PKM or Csk did not affect EGF- or nerve-growth-factor-induced MAP kinase activation in PC12 (data not shown). Taken together, these experiments reveal a specific role for Pyk2 and Src in linking G-protein-coupled receptors with MAP kinase activation in PC 12 cells.

We further analysed agonist-induced MAP kinase activity in PC12 cell lines that stably overexpress a dominant interfering mutant of Grb2 lacking the N-terminal SH3 domain (Grb2 DN-SH3) or in PC12 cells that stably overexpress the proline-rich tail of Sos (Sos-CT)^{14,15}. Overexpression of Grb2 DN-SH3 in PC12 cells completely blocked LPA- or bradykinin-induced MAP kinase activation (Fig. 4c). Overexpression of Sos-CT strongly reduced MAP kinase activation in response to LPA and bradykinin stimulation (Fig. 4c). However, activation of Pyk2 or Src was not affected by the dominant interfering mutants of Grb2 and Sos, confirming that Pyk2 and Src act upstream of Grb2 and Sos in the cascade of events leading to MAP kinase activation (data not shown).

Our results show that Pyk2 can link both G_i - or G_q -protein-coupled receptors with the MAP kinase signalling pathway in PC12 cells. Phosphorylation of Tyr 402 of Pyk2 leads to binding of the SH2 domain of Src and subsequent Src activation in response to either G_i - or G_q -protein-coupled receptors. Overexpression of activated Src (Y527F) in PC12 cells induces tyrosine phosphorylation of Pyk2, but does not stimulate Pyk2 kinase activity (data not shown). Tyrosine phosphorylation of Pyk2 might therefore be partly mediated by Src, thus generating docking sites for additional signalling proteins that are recruited by Pyk2. We have previously shown that recruitment of Grb2/Sos as a result of activation of Pyk2 can occur directly or indirectly through tyrosine phosphorylation of Shc⁵. The results presented here indicate that dominant interfering mutants of Grb2 or Sos strongly inhibit LPA- or bradykinin-induced activation of MAP kinase, in agreement with findings that a Sos-deletion mutant or a dominant interfering mutant of Shc can block LPA- or thrombin-induced activation of MAP kinase, respectively^{4,16}. Together, these experiments underscore the central role of the Shc/Grb/Sos complex in mediating MAP kinase activation not only by receptor tyrosine kinases, but also by G_i - and G_q -protein-coupled receptors. In avian B cells, both Lyn and Syk are essential for activation of MAP kinase by G_i - and G_q -protein-coupled receptors³, so a combination of different protein tyrosine kinases in different tissues and cell types may link G-protein-coupled receptors with the MAP kinase signalling pathway. Src family protein tyrosine kinases, which are expressed in every cell type and tissue, appear to be a common and important component of this pathway, acting together with cell-type-specific protein tyrosine kinases, such as Pyk2 in PC12 cells or Syk in avian B cells, to bring about a cell-type-specific signal for linking G-protein-coupled receptors with the MAP kinase signalling pathway and hence the transcriptional machinery. □

Methods

Cells and transfections. PC12 cells, PC12 cells overexpressing a Grb2 N-SH3 deletion mutant (Grb2 DN-SH3) or the deletion mutant of Sos (proline-rich tail of Sos, Sos CT), and 293T cells were all grown as described^{13,17}. For transient transfection of PC12 cells, cells were grown to 60% confluence on 10-cm plates and transfected with 20 µg of pRK5, PKM or Csk plasmids using lipofectamine reagent (Gibco BRL). For PKM + Csk transfections, 20 µg PKM and Csk plasmids were used. For β -galactosidase (β -gal) assays, 15 µg of a β -gal transgene reporter vector were used. Cells were transfected for 12 h and lysed after 36 h, yielding ~30% of transfected viable cells as determined by β -gal assay. 293T cells were grown to 50% confluence on 10-cm plates and transiently transfected with 8 µg pRK5, 5 µg Pyk2 + 3 µg pRK5, 5 µg Pyk2-Y402F + 3 µg pRK5, and 8 µg Pyk2 kinase-negative mutant (PKM) using lipofectamine reagent (Gibco BRL).

Cell lysis, immunoprecipitations and immunoblotting. Cells lysis, immunoprecipitation and immunoblotting were done as described^{13,17}. For Figs 2 and 3, cells were lysed in modified RIPA buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.3% sodium deoxycholate, 0.1% Nonidet P-40, 1.5 mM MgCl₂, 1 mM EDTA, 0.2 mM EGTA, 1 mM sodium orthovanadate, 20 mM sodium fluoride, 25 µM zinc chloride, 1 mM phenylmethylsulphonyl fluoride, 10 µg ml⁻¹ aprotinin and 10 µg ml⁻¹ leupeptin). Polyclonal Pyk2 antibodies were raised against fusion proteins containing amino acids 684 to 1,009 (cat. no. 598) or amino acids 684 to 762 (cat. no. 600) of human Pyk2 expressed as a GST-fusion protein in pGEX-2T (Pharmacia Biotech). Anti-Pyk2 antibodies (no. 600) were crosslinked to protein-A beads (Zymed) with dimethylpimelimidate (Pierce) as described¹³. Antibodies against Src (Ab-1) were from Oncogene Sciences; affinity-purified anti-pY416⁸⁰ polyclonal antibodies were kindly provided by A. Laudano. Antibodies against Pyk2 (no. 598) were purified on a GST column and used at a dilution of 1:300 in TBS containing 5% bovine serum albumin (Intergen). Antibodies against phosphotyrosine (anti-pTyr) were used as described¹⁷. The preparation and purification of a GST fusion protein containing the SH2 domain of Src and the binding assay have been described¹⁸.

In vitro mutagenesis. The mutagenic oligonucleotide (GAGTCAGACATCTTC

GCAGAGATTCCC) and the *trans* oligonucleotide (GAATTCGATATCACGCGTTGGCCGCCATGGC) were used to convert Tyr 402 to Phe of Pyk2 using a Clontech kit⁵; the mutation was confirmed by DNA sequencing.

Kinase assays. MAP kinase was assayed *in vitro* using MBP as a substrate^{5,17}. The results shown in Fig. 4b were calculated as a percentage of MAP kinase activity relative to activation in mock-transfected cells. Data were normalized for transfection efficiency by multiplying the increase in MAP kinase activity for each point by the transfection efficiency determined for expression of a β -gal transgene reporter.

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CORRESPONDENCE and requests for materials should be addressed to J.S.

Assembly of microtubule-associated protein tau into Alzheimer-like filaments induced by sulphated glycosaminoglycans

M. Goedert, R. Jakes, M. G. Spillantini*, M. Hasegawa, M. J. Smith & R. A. Crowther

MRC Laboratory of Molecular Biology, Hills Road, Cambridge CB2 2QH, UK

THE paired helical filament (PHF) is the major component of the neurofibrillary deposits that form a defining neuropathological characteristic of Alzheimer's disease (reviewed in refs 1,2). PHFs are composed of microtubule-associated protein tau, in a hyperphosphorylated state^{3–8}. Hyperphosphorylation of tau results in its inability to bind to microtubules^{9,10} and is believed to precede PHF assembly¹¹. However, it is unclear whether hyperphosphorylation of tau is either necessary or sufficient for PHF formation. Here we show that non-phosphorylated recombinant tau isoforms with three microtubule-binding repeats form paired helical-like filaments under physiological conditions *in vitro*, when incubated with sulphated glycosaminoglycans such as heparin or heparan sulphate. Furthermore, heparin prevents tau from binding to microtubules and promotes microtubule disassembly. Finally, we show that heparan sulphate and hyperphosphorylated tau coexist in nerve cells of the Alzheimer's disease brain at the earliest known stages of neurofibrillary pathology. These findings, with previous studies which show that heparin stimulates

tau phosphorylation by a number of protein kinases^{12–14}, indicate that sulphated glycosaminoglycans may be a key factor in the formation of the neurofibrillary lesions of Alzheimer's disease.

Sulphated glycosaminoglycans were used to stimulate phosphorylation of recombinant tau, and high-molecular-weight aggregates of tau formed in a glycosaminoglycan-dependent, but phosphorylation-independent, manner. Numerous filaments were observed by electron microscopy, with different morphologies depending on the number of microtubule-binding repeats. Tau isoforms with three repeats gave filaments with a typical paired helical morphology (Fig. 1a), whereas tau isoforms with four repeats gave filaments with a straight appearance (Fig. 1b). Tau protein that had been phosphorylated by p42 mitogen-activated protein (MAP) kinase or neuronal Cdc2-like kinase (NCLK) before incubation with heparin or heparan sulphate, formed filaments that were indistinguishable from those formed by non-phosphorylated tau, indicating that tau phosphorylation is neither stimulatory nor inhibitory to *in vitro* filament formation (Fig. 1c). On co-incubation of tau with sulphated glycosaminoglycans and p42 MAP kinase or NCLK, filaments with the same morphology were formed.

Immunoelectron microscopy indicated that the paired helical-like filaments were decorated by antibodies directed against the amino- and carboxy-termini of tau, but not by an antibody against the microtubule-binding repeat region (Fig. 2). These results indicate that in the filaments the repeat region of tau is inaccessible to the antibody, and are identical to those obtained previously with PHFs from Alzheimer's disease brain⁷. In the presence of heparin, each of the six recombinant human brain tau isoforms formed either paired helical or straight filaments. Similar results were obtained with truncated tau proteins consisting of three or four microtubule-binding repeats, and with proteins comprising the repeat region and the C terminus of tau. By contrast, a truncated form, comprising the N-terminal half of tau up to but excluding the repeat region, failed to give filaments (data not shown). Therefore, the microtubule-binding repeat region of tau is essential for sulphated glycosaminoglycan-induced filament

* Present address: MRC Cambridge Centre for Brain Repair, University of Cambridge, Robinson Way, Cambridge CB2 2PY, UK.

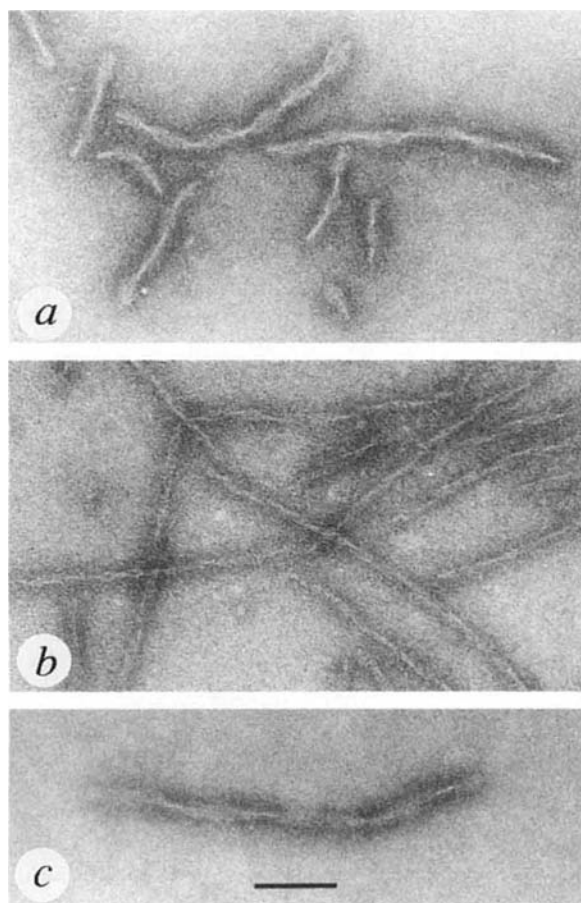


FIG. 1 Sulphated glycosaminoglycan-induced filament assembly of human tau protein. *a*, The three-repeat containing 381 amino-acid isoform (htau37), or *b*, the four-repeat containing 441 amino-acid isoform (htau40) was incubated with heparin. *c*, htau37 was phosphorylated with NCLK before the addition of heparin. Note the presence of paired helical-like filaments in *a* and *c* and of straight filaments in *b*. Scale bar, 100 nm.

formation. Three microtubule-binding repeats of tau form the core of the PHF from Alzheimer's disease brain^{4,15}, supporting the evidence for a similar organization of the two types of filament. Moreover, the dimensions of tau filaments formed in the presence of sulphated glycosaminoglycans were similar to filaments extracted from Alzheimer's disease brain, with a diameter of ~20 nm for twisted, and ~15 nm for straight filaments and a crossing-over spacing of ~80 nm for paired helical-like filaments, although their twist was less regular than in Alzheimer filaments.

At optimal concentrations of tau and heparin, paired helical-like filaments started to form after ~5 h at 30 °C reaching a maximum after 48 h. Filament formation was dependent on the absolute concentration of tau and on the tau:sulphated glycosaminoglycan ratio; paired helical and straight filaments formed at concentrations of tau above 40 μ M, and a tau:sulphated glycosaminoglycan molar ratio of approximately 4:1. The number of filaments, but not their morphology, correlated with the degree of glycosaminoglycan sulphation; the largest number was obtained following incubation of tau with heparin, with fewer filaments after incubation with heparan sulphate. Chondroitin sulphate and dermatan sulphate gave the smallest number of filaments, and hyaluronic acid gave no filaments. Similarly, no filaments were obtained following incubation of tau with poly-L-glutamic acid, indicating that filament formation does not result from a simple charge interaction.

The heparin-binding properties of tau were investigated using heparin-Sepharose. The six recombinant tau isoforms bound to heparin-Sepharose in a salt-dependent manner, eluting at 0.3–0.5 M NaCl (Fig. 3*a*). Truncated tau consisting of three or four repeats, or comprising the repeat region and the C terminus, also bound to heparin-Sepharose, as did a fragment containing the N-terminal half of tau up to but excluding the repeat region (data not shown). This demonstrates the presence of multiple heparin-binding sites, distributed throughout the tau sequence. Similarly, recombinant tau phosphorylated with p42 MAP kinase or NCLK was able to bind to heparin-Sepharose, indicating that phosphorylation did not significantly affect the ability of tau to bind to heparin. This binding is probably mediated through electrostatic interactions between the negative charges in heparin and positive charges in tau. Tau is positively charged in the repeat region and in the region upstream of the repeats, although the N-terminal 125 amino acids are negatively charged. In contrast to tau, tubulin failed to bind to heparin-Sepharose (Fig. 3*a*).

We next examined the influence of heparin on the ability of tau to bind to microtubules and to promote microtubule assembly. The presence of heparin prevented tau from binding to taxol-stabilized microtubules (Fig. 3*b*). Moreover, microtubules

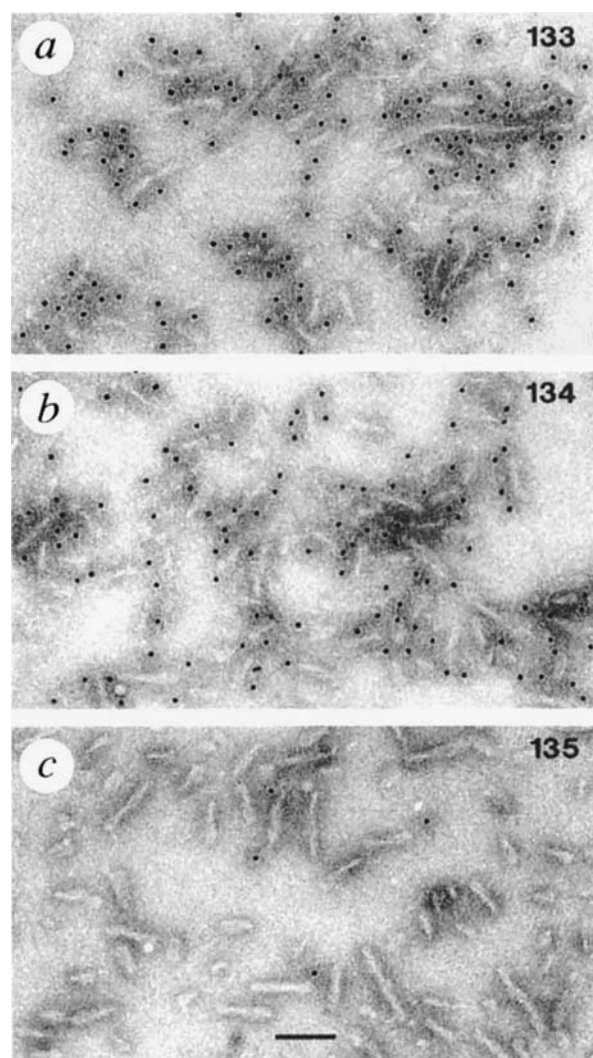


FIG. 2 Immunoelectron microscopy of paired helical-like filaments formed following incubation of htau37 with heparin, using antibodies directed against *a*, the N terminus (antiserum 133); *b*, the C terminus (antiserum 134); and *c*, the microtubule-binding repeats (antiserum 135) of tau. Gold labelling is seen with 133 and 134, but not with 135, as for Alzheimer filaments⁷. Scale bar, 100 nm.

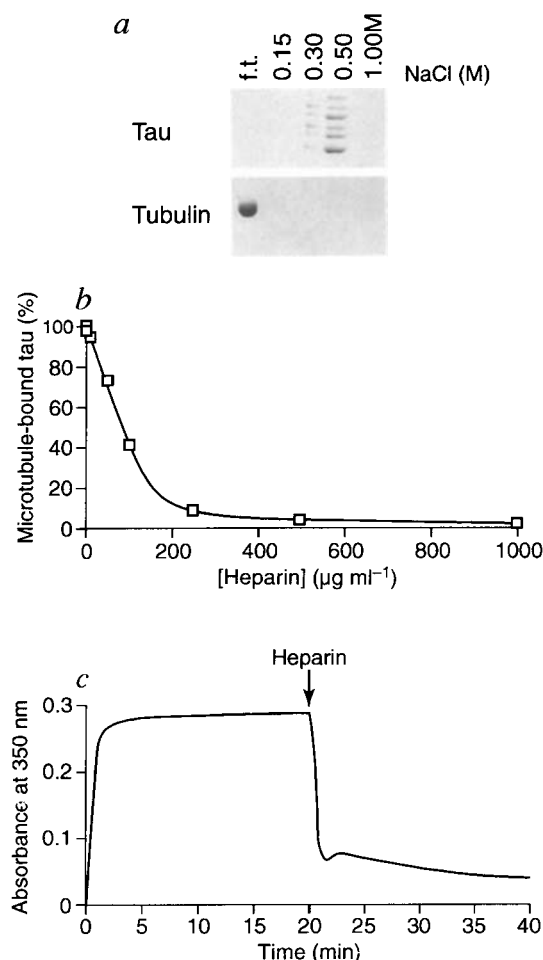


FIG. 3 a, Binding of tau proteins to heparin-Sepharose; f.t., flow-through. b, Effect of heparin on the binding of tau to microtubules. c, Effect of heparin on tau-promoted microtubule assembly.

assembled in the presence of tau rapidly depolymerized when heparin was added (Fig. 3c). Microtubules assembled from high concentrations of tubulin without tau did not disassemble when heparin was added (data not shown). These results demonstrate that heparin prevents tau from binding to microtubules and from promoting microtubule assembly and stability.

Electron microscopic studies indicated that intracellular staining of heparan sulphate in neurofibrillary lesions¹⁶⁻¹⁹ is associated with cytoplasmic PHFs^{16,17}. However, it was not known whether heparan sulphate and hyperphosphorylated tau coexist in the earliest known stages of neurofibrillary pathology, where nerve cells stain diffusely with phosphorylation-dependent anti-tau antibodies¹¹. We therefore performed single- and double-labelling immunohistochemistry on hippocampal formation from Alzheimer's disease brain, using the anti-heparan sulphate antibody 10E4 (ref. 20) and the phosphorylation-dependent anti-tau antibody AT8 (refs 21, 22) (Fig. 4). A subset of pyramidal cells was found to be strongly immunoreactive with 10E4 (Fig. 4a); these included cells with overt neurofibrillary tangles, as well as non-tangle-bearing cells. Double-labelling immunohistochemistry showed that nerve cells that stained diffusely with AT8, also stained with 10E4 (Fig. 4b-f). In most cells, the brown 10E4 and the blue AT8 staining were inter-mixed. However, in some nerve cells, the heparan sulphate staining was more extensive than the tau staining (Fig. 4c, f, arrows). In particular, small patches of tau staining were observed in some pyramidal cells with diffuse heparan sulphate staining (Fig. 4c, d, arrows). Moreover, some tau-negative nerve cells were heparan sulphate-positive, suggest-

ing that the accumulation of heparan sulphate precedes hyperphosphorylation of tau in these cells. As shown previously¹⁶⁻¹⁹, tau and heparan sulphate immunoreactivities coexisted in nerve cells with overt neurofibrillary lesions, and amyloid plaques were stained with the heparan sulphate antibody. The immunostaining was specific; heparan sulphate staining was absent following heparitinase treatment of the tissue sections and the tau staining was absent following pre-adsorption of the antibody with phosphorylated tau. In age-matched control brains, only very small numbers of cells stained with 10E4 or AT8.

The findings presented here indicate that interactions between tau protein and sulphated glycosaminoglycans may lead to the formation of the neurofibrillary lesions of Alzheimer's disease. These lesions consist of PHFs and related straight filaments²³, containing hyperphosphorylated tau protein that is unable to bind to microtubules^{3-6,9,10}. Sulphated glycosaminoglycans stimulate the phosphorylation of tau by a number of protein kinases in a substrate-dependent manner¹²⁻¹⁴. Under physiological conditions, a phosphorylation-independent interaction between full-length recombinant tau and sulphated glycosaminoglycans leads to the formation of filaments closely resembling those found in the Alzheimer's disease brain. In the presence of sulphated glycosaminoglycans, three-repeat-containing tau isoforms give rise to paired helical-like filaments, whereas four-repeat-containing isoforms form straight filaments, thus suggesting an explanation for the two tau assemblies present in Alzheimer's disease brain. Previous studies under non-physiological conditions showed that paired helical-like filaments formed only from fragments of tau consisting of three microtubule-binding repeats²⁴⁻²⁶ and to a limited extent from the full-length molecule²⁷. We also show here that the interaction between tau and sulphated glycosaminoglycans prevents tau from binding to microtubules and from promoting microtubule assembly. Moreover, heparan sulphate and tau immunoreactivities co-localize in nerve cells in Alzheimer's disease brain before the formation of overt neurofibrillary lesions.

Our findings provide a simple assay for the identification of compounds that interfere with PHF formation. Future experiments will investigate whether other molecules implicated in the aetiology and pathogenesis of Alzheimer's disease, such as amyloid A β (ref. 28), apolipoprotein E²⁹ and the presenilins³⁰, influence the interactions between tau and sulphated glycosaminoglycans. An increase in sulphated glycosaminoglycans within the cytoplasm of nerve cells may trigger the hyperphosphorylation of tau, destabilize microtubules and induce assembly of PHFs. Interactions between sulphated glycosaminoglycans and tau may thus be the central event in the development of neurofibrillary pathology in Alzheimer's disease. □

Methods

Filament assembly. Tau protein isoforms were expressed in *Escherichia coli* and purified as described³¹, except that the boiling step was omitted, and ammonium sulphate precipitation and Sephacryl S-200 gel filtration steps were added. Purified htau37 and htau40 (3 mg ml^{-1}) were incubated with heparin ($200 \mu\text{g ml}^{-1}$; BDH) in $25 \mu\text{l}$ of 30 mM MOPS, 1 mM 4-(2-aminoethyl)benzenesulphonylfluoride (AEBSF, Calbiochem), pH 7.4, at 30°C for 48 h. Samples were examined by electron microscopy and processed for immunoelectron microscopy⁷. In some experiments, htau37 was phosphorylated with MAP kinase or NCLK³², before addition of heparin. Filaments were not observed with unphosphorylated or phosphorylated tau protein alone. Sulphated glycosaminoglycans on their own also failed to form filaments. Similar results were obtained in over 50 separate experiments using tau isoforms and heparan sulphate or heparin from different commercial sources, with the relative molecular masses (M_r) of heparin ranging from 3K to 30K.

Microtubule binding and assembly. An equal mixture of the six recombinant human brain tau isoforms ($60 \mu\text{g}$ total) or $20 \mu\text{g}$ bovine brain tubulin (Cytoskeleton) were applied to a heparin-Sepharose (Pharmacia) column ($4.5 \times 8 \text{ mm}$) in 30 mM MOPS, pH 7.4. The column was eluted with 10 column volumes of buffer containing 0 (flow-through), 0.15 M, 0.3 M, 0.5 M and 1.0 M NaCl. Aliquots were subjected to SDS-PAGE and stained with Coomassie blue. For microtubule binding studies, recombinant htau40 ($4 \mu\text{M}$, 0.18 mg ml^{-1}) was incubated with different concentrations of heparin (0, 2, 20, 100, 200, 500,

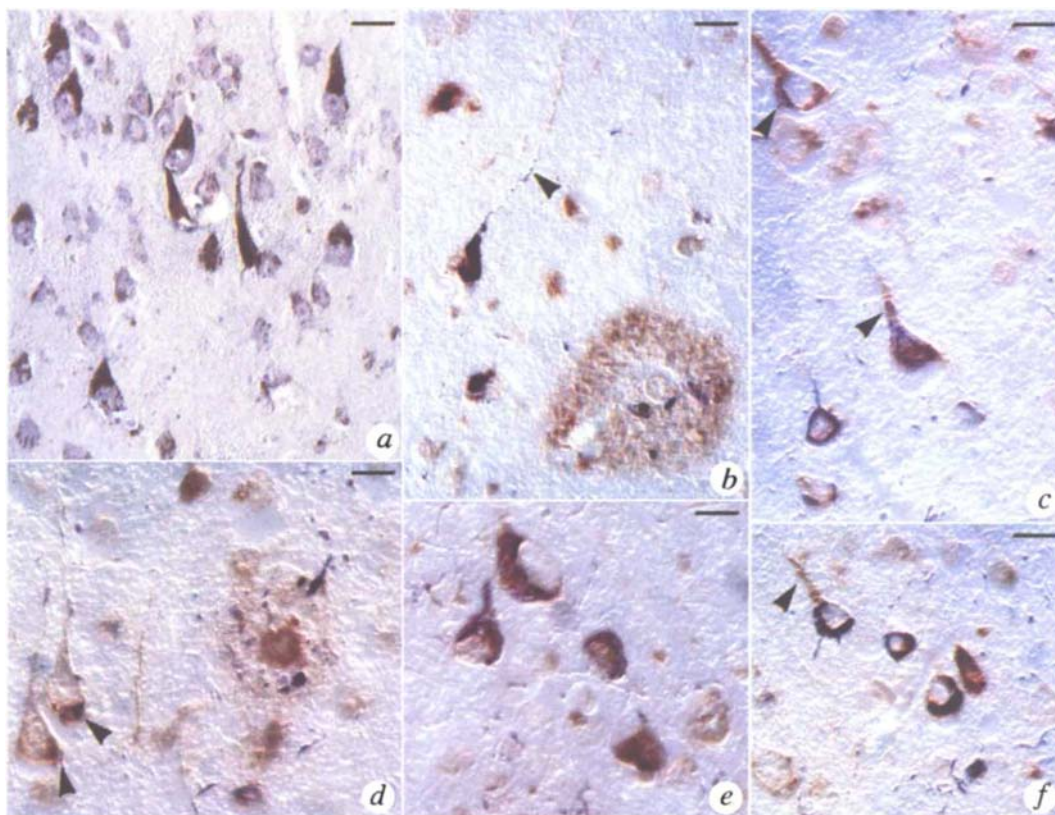


FIG. 4 Staining of hippocampal formation from Alzheimer's disease brain with: a, the anti-heparan sulphate antibody 10E4; and b–f, double-staining with 10E4 and the phosphorylation-dependent anti-tau antibody AT8. 10E4 staining is shown in brown and AT8 staining in blue (Nomarski optics). Note staining of some pyramidal cells with 10E4 (a) and double-

labelling of many nerve cells (b–f). Pyramidal cells with partially double-labelled cell bodies or neurites are arrowed (b, c, d and f). The section in a was counterstained with thionin, but there was no counterstaining in b–f. Scale bars: 45 μ m in a, 34 μ m in b, 30 μ m in c, 24 μ m in d and e, and 40 μ m in f.

1,000, 2,000 μ g ml⁻¹) in assembly buffer (80 mM PIPES, 1 mM MgCl₂, 1 mM EGTA, 1 mM dithiothreitol, 1 mM GTP, pH 6.8) for 10 min at 37 °C, then added to 10 μ M taxol-stabilized microtubules and incubated for a further 20 min. Following ultracentrifugation, aliquots of supernatants (free tau) and pellets (microtubule-bound tau) were subjected to SDS–PAGE. Protein concentrations were estimated by scanning the gels with a Molecular Dynamics computing densitometer (Model 300A), and are expressed as percentage of tau bound to microtubules in the absence of heparin (taken as 100%). For microtubule assembly, recombinant htau40 (2 μ M) was incubated with tubulin (10 μ M) in assembly buffer at 37 °C. After 20 min, heparin (100 μ g ml⁻¹) was added and incubated for a further 20 min. Polymerization and depolymerization of microtubules were monitored by measuring the absorbance at 350 nm. Addition of heparin led to rapid disassembly of microtubules.

Immunohistochemistry. Deparaffinized 4- μ m sections through the hippocampal formation from three Alzheimer's disease patients (76, 79 and 80 years of age) were used for single- and double-labelling immunohistochemistry³³. Antibody 10E4 (Seikagaku Corporation) was used at a dilution of 1:250 and antibody AT8 (Innogenetics) at 1:500. In some experiments, the order of addition of the primary antibodies was inverted and the first or second primary antibody was omitted. As a control, tissue sections were incubated for 3 h at 37 °C with 0, 2.5, 5 or 10 mU heparitinase (Seikagaku Corporation) in 0.5 ml 50 mM HEPES buffer, pH 7.0, containing 100 mM NaCl, 1 mM CaCl₂, 10 μ g ml⁻¹ BSA and 1 mM phenylmethyl sulphonylfluoride (PMSF; Sigma). After washing in PBS, the sections were incubated with antibody 10E4, as described above. Staining with 10E4 was greatly reduced with 2.5 mU heparitinase and abolished with 5 and 10 mU of the enzyme, whereas AT8 staining was unaffected by heparitinase treatment. AT8 staining was abolished following incubation of the diluted antibody with 10 μ M phosphorylated recombinant tau protein.

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CORRESPONDENCE and requests for materials should be addressed to M.G. (e-mail: mg@mrc-lmb.cam.ac.uk).

Structure of a unique binuclear manganese cluster in arginase

Zoltan F. Kanyo*, Laura R. Scolnick*, David E. Ash† & David W. Christianson*

* Department of Chemistry, 231 S. 34th Street, University of Pennsylvania, Philadelphia, Pennsylvania 19104-6323, USA

† Department of Biochemistry, Temple University School of Medicine, Philadelphia, Pennsylvania 19140, USA

EACH individual excretes roughly 10 kg of urea per year, as a result of the hydrolysis of arginine in the final cytosolic step of the urea cycle¹. This reaction allows the disposal of nitrogenous waste from protein catabolism, and is catalysed by the liver arginase enzyme². In other tissues that lack a complete urea cycle, arginase regulates cellular arginine and ornithine concentrations for biosynthetic reactions³, including nitric oxide synthesis: in the macrophage, arginase activity is reciprocally coordinated with that of NO synthase to modulate NO-dependent cytotoxicity⁴⁻⁹. The bioinorganic chemistry of arginase is particularly rich because this enzyme is one of very few that specifically requires a spin-coupled Mn^{2+} - Mn^{2+} cluster for catalytic activity *in vitro* and *in vivo*¹⁰. The 2.1 Å-resolution crystal structure of trimeric¹¹ rat liver arginase reveals that this unique metal cluster resides at the bottom of an active-site cleft that is 15 Å deep. Analysis of the structure indicates that arginine hydrolysis is achieved by a metal-activated solvent molecule which symmetrically bridges the two Mn^{2+} ions.

The overall fold of the arginase monomer belongs to the α/β protein class with a globular structure of approximate dimensions 40 × 50 × 50 Å. One side of the active-site cleft is partially defined by the central 8-stranded β -sheet, and the metal binding site is located on one edge of the β -sheet. Metal ligands are located immediately adjacent to helix C, strand 4, and strand 7 (Fig. 1). Assembly of the trimer, which has a relative molecular mass of 105,000 (M_r 105K), excludes 2,080 Å² of surface area from solvent at each monomer-monomer interface. A majority (54%) of intermonomer contact is mediated by a novel 'S'-shaped oligomerization motif at the carboxy terminus (Fig. 1), and the conformation of this segment is stabilized by numerous intermonomer van der Waals interactions, hydrogen bonds and salt links.

The binuclear manganese cluster is located at the base of a 15-Å-deep active-site cleft in each monomer (Fig. 2). The metal ion

that is more deeply situated in the active-site cleft (designated Mn_A^{2+}) is coordinated by His 101 (N δ), Asp 124 (O δ 1), Asp 128 (O δ 1), Asp 232 (O δ 1) and a solvent molecule, with square pyramidal geometry. The solvent molecule bridges both metal ions and also donates a hydrogen bond to Asp 128 (O δ 2-O separation = 2.8 Å). The second metal ion (designated Mn_B^{2+}) is coordinated by His 126 (N δ), Asp 124 (O δ 2), Asp 232 (O δ 1), Asp 234 (bidentate O δ 1 and O δ 2) and the bridging solvent molecule in distorted octahedral fashion. The Mn_A^{2+} - Mn_B^{2+} separation is 3.3 Å. All metal ligands except for Asp 128 make hydrogen-bond interactions with other protein residues, and these interactions contribute to the stability of the metal binding site¹².

Three different types of bridging metal ligands facilitate the observed spin coupling¹⁰ between Mn_A^{2+} and Mn_B^{2+} (Fig. 2). The carboxylate side chain of Asp 124 is a *syn-syn* bidentate bridging ligand¹³, with O δ 1 coordinated to Mn_A^{2+} and O δ 2 coordinated to Mn_B^{2+} . The carboxylate side chain of Asp 232 is a monodentate bridging ligand¹³, with O δ 1 coordinated to both Mn_A^{2+} and Mn_B^{2+} with *anti*- and *syn*-coordination stereochemistry, respectively (the 'dangling' oxygen O δ 2 is 3.8 Å away from Mn_B^{2+}). Finally, the solvent molecule bridges both manganese ions symmetrically, with Mn_A^{2+} -O and Mn_B^{2+} -O separations of 2.4 Å.

The arginase structure is the first atomic resolution structure of a functional metalloenzyme that has a specific catalytic and physiological requirement for two Mn^{2+} ions. In catalysis, arginase does not use transition metals promiscuously (unlike other metalloenzymes in which Mn^{2+} might be substituted for Mg^{2+} or other metal ions, or vice versa), and the identity and oxidation state of the manganese cluster is conclusively established by electron paramagnetic resonance spectroscopy¹⁰. The catalytic metal requirement is rooted in the preferred geometry of manganese coordination, which properly orients the metal-bridging solvent molecule for catalysis. As a metal-bridging solvent molecule must satisfy the coordination preferences of two manganese ions simultaneously, its position, and therefore its optimal catalytic activity, would be highly sensitive to the substitution of one or both Mn^{2+} ions. Coordination of a catalytic group to two metals rather than one may enhance the dependence of optimal catalytic activity on proper metal selectivity.

Only two other polar residues are found in the immediate active site: Glu 277 and His 141. Glu 277 is located deep in the active-site cleft 4.5 Å away from Mn_A^{2+} . A roughly 20° rotation about χ_1 of this side chain would yield an ideal salt link with the substrate guanidinium group. Moreover, this interaction would position the electrophilic guanidinium carbon of the substrate directly over the metal-bridging solvent molecule, which is likely to be nucleophilic hydroxide ion in the active catalyst (Fig. 3). It is unlikely that

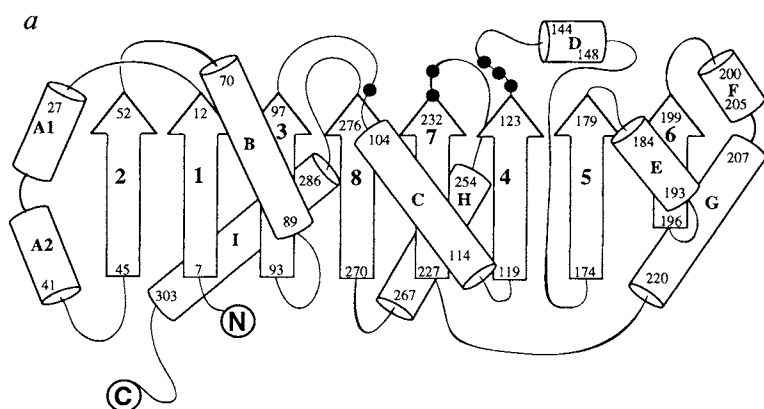


FIG. 1 a, Topology diagram of arginase. Relative locations of metal ligands are indicated by solid circles. b, Ribbon-plot of the arginase trimer; the Mn^{2+} - Mn^{2+} cluster in the active site of each monomer is represented by a pair of spheres. At the C terminus of each monomer, the polypeptide chain forms an unusual 'S'-shaped oligomerization motif. Figure generated with MOLSCRIPT²⁵ and Raster3D^{26,27}.

the deprotonated substrate guanidinium group binds directly to the metal(s) because of its high pK_a of 13.5. Furthermore, site-directed mutagenesis studies show that substrate K_m values are not perturbed by variations in the metal cluster (including metal depletion), indicating that a substrate-metal interaction does not occur in the Michaelis complex¹⁴.

The side chain of His 141 is located about half-way out of the active-site cleft. Interestingly, arginase with asparagine substituted for histidine at position 141 (His 141 $\rightarrow$ Asn arginase) retains roughly 10% residual activity compared with the wild-type enzyme¹⁴. Given its location 4.2 Å away from the metal-bridging solvent molecule, it is possible that His 141 is a proton shuttle in catalysis (Fig. 3), mediating proton transfer to and from bulk solvent. Direct proton transfer with bulk solvent may be operative in the absence of His 141, which could account for the significant residual catalytic activity of His 141 $\rightarrow$ Asn arginase. A proton shuttle function for His 141 of arginase would be analogous to that recently reviewed for His 64 in the zinc metalloenzyme carbonic anhydrase II (ref. 15).

On opposite sides of the active-site lip, charged residues are found which may contribute to the exquisite specificity of substrate recognition. Structure-activity relationships for arginine analogues indicate that electrostatic interactions with α -substituents of the substrate are critical for catalysis: deletion of the α -carboxylate group or the α -amino group results in

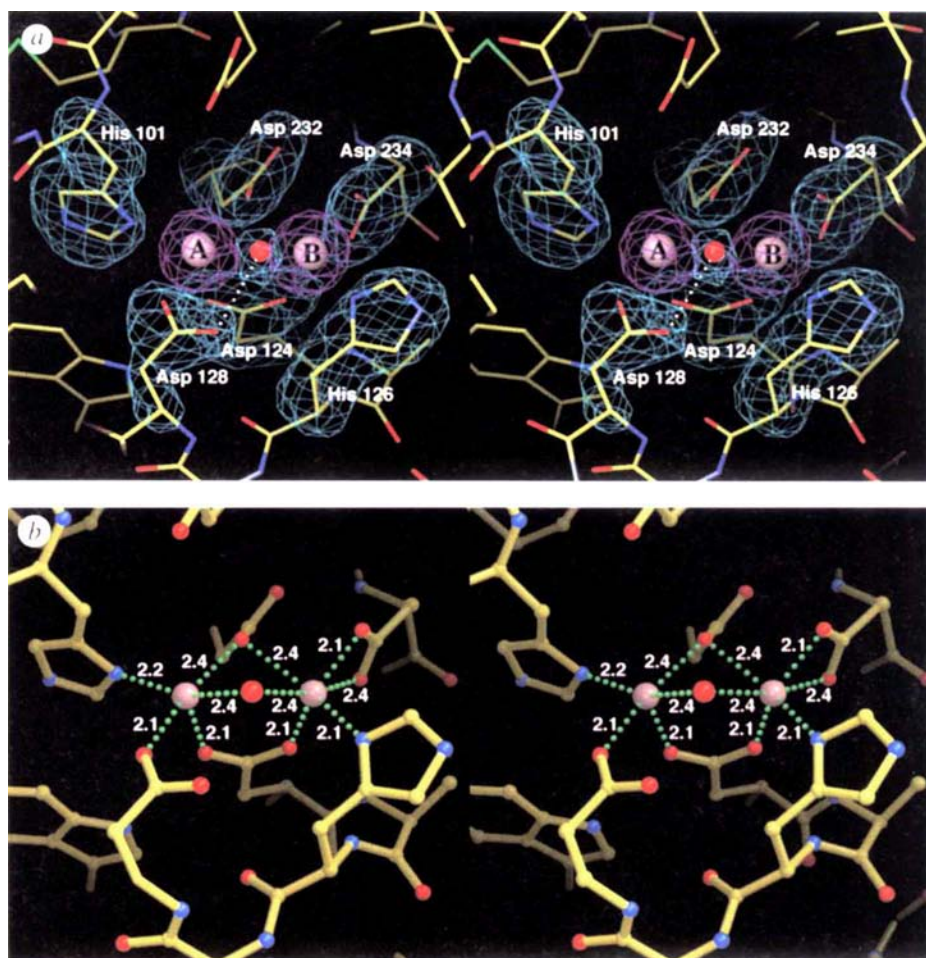


FIG. 2 *a*, OMIT maps of the binuclear manganese cluster calculated with Fourier coefficients $|F_o| - |F_c|$ and phases derived from the final model less the atoms of metal ligands (blue map, contoured at 3σ), or metal ions Mn^{2+} and Mn^{3+} (magenta map, contoured at 10σ). The hydrogen bond between O δ 2 of Mn^{2+} ligand Asp 128 and bridging solvent (red sphere) is indicated by a dashed line. *b*, Manganese coordination interactions (Å). The coordination geometry of Mn^{2+} is square pyramidal, and that of Mn^{3+} is octahedral but distorted owing to the bidentate coordination of Asp 234.

TABLE 1 Data collection and refinement statistics

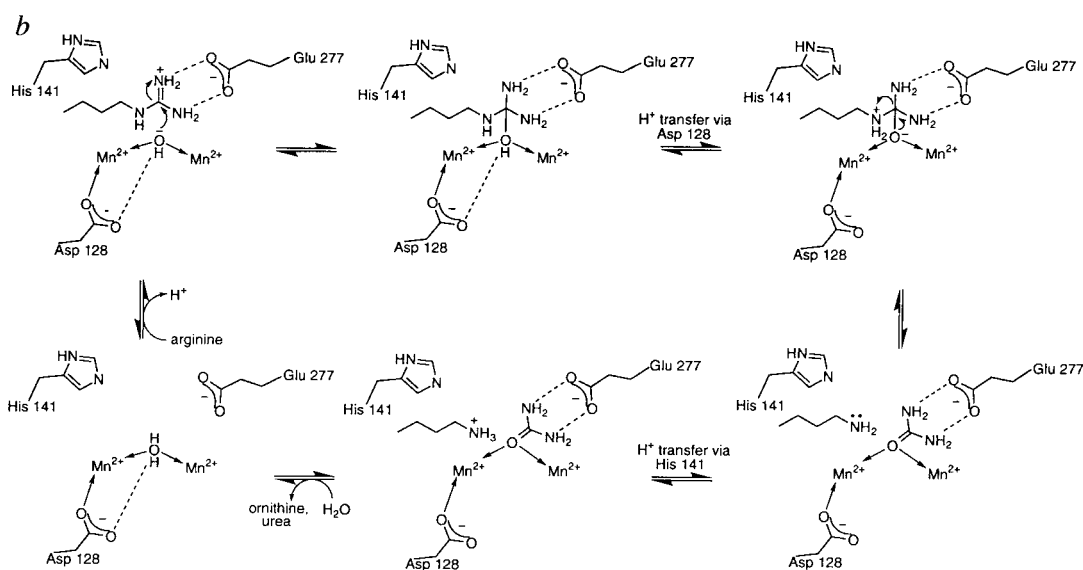
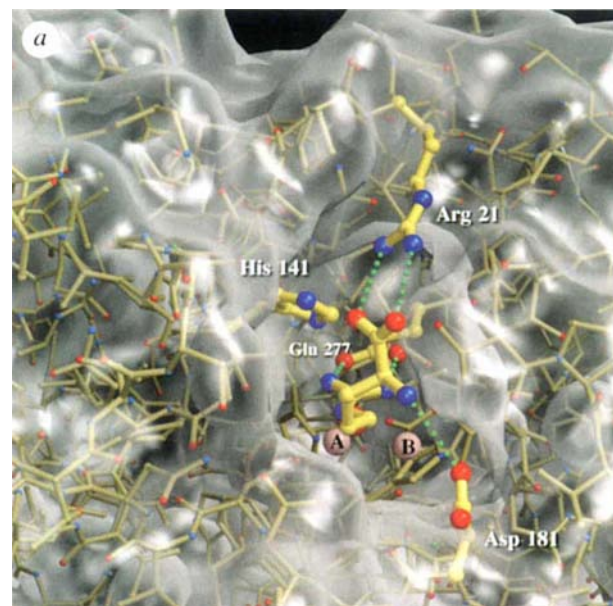
Data set	Resolution (Å)	Reflections measured/unique	Completeness (%) overall/outer shell	R_{merge}^* overall/outer shell	$R_{\text{iso}}^\dagger$	Number of sites	Phasing‡ power	Overall figure of merit		
Native	2.1	147,454/46,162	91.6/58.3	0.058/0.365	—	—	—	—		
Thimerisol	3.0	39,030/17,363	93.7/96.5	0.063/0.226	0.199	6	1.51	—		
YbCl ₃	3.0	29,644/15,182	81.9/86.0	0.042/0.146	0.143	6	1.46	—		
HgAc ₂	3.0	26,788/16,202	87.2/91.6	0.089/0.272	0.127	6	1.78	—		
0.496										
Refinement										
Protein	Atoms	Mn ²⁺ ions	Solvent molecules	Resolution (Å)	No. reflections work/free	$R/R_{\text{free}}^\S$	R.m.s. deviations			
							Bonds	Angles	Dihedrals	Impropers
Native	7,167	6	233	2.1	47,913/2,502	0.179/0.229	0.011 Å	1.6°	24.7°	1.6°

* $R_{merge} = \sum |I_i - \langle I_i \rangle| / \sum \langle I_i \rangle$, where I_i is the intensity measurement for reflection i , and $\langle I_i \rangle$ is the mean intensity calculated for reflection i from replicate data.

$^\dagger R_{iso} = \sum \|F_{PH}\| - \|F_P\| / \sum \|F_P\|$, where F_{PH} and F_P are the derivative and native structure factors, respectively.

‡ Phasing power = $\langle F_n \rangle / E$ where $\langle F_n \rangle$ is the root-mean-square heavy-atom structure factor and E is the residual lack of closure error.

$^\S R = \sum \|F_o\| - \|F_c\| / \sum \|F_o\|$, where R and R_{free} are calculated using the working and free reflection sets, respectively. The free reflections (5% of the total) were held aside throughout refinement.



10^2 – 10^5 -fold reductions in k_{cat}/K_m (ref. 16). Inspection of the arginase active site indicates that the positively charged side chain of Arg21 may interact with the negatively charged α -carboxylate group of the substrate, and the negatively charged side chain of Asp 181 may interact with the positively charged α -amino group of the substrate. A model of substrate binding is presented in Fig. 3.

The features outlined above are incorporated into a proposal for the mechanism of arginine recognition and hydrolysis by metal-activated solvent (Fig. 3). The ionization of metal-bound water probably reflects the apparent pK_a of 7.9 observed in the pH–rate profile¹⁷. In the first step of the hydrolytic mechanism, Asp 128 stabilizes the metal-bridging hydroxide ion with a hydrogen bond during nucleophilic attack at the guanidinium carbon of arginine. The resulting tetrahedral intermediate collapses upon proton transfer to the amino group of ornithine, and this proton transfer is probably mediated by Asp 128. Subsequently, we propose that His 141 shuttles a proton from bulk solvent to the ε -amino group of ornithine before product dissociation. Upon product dissociation, a water molecule displaces urea. Metal coordination facilitates the ionization of this water molecule to regenerate a nucleophilic hydroxide ion. Here, proton transfer to bulk solvent may again be mediated by shuttle-group His 141.

It is instructive to consider the chemical function of arginase not only within the context of mammalian nitrogen metabolism, but also within the greater context of the biosphere. In the nitrogen cycle, two binuclear metalloenzymes of different tertiary structure are prominent: Mn_2^{2+} -arginase and Ni_2^{2+} -urease. The former enzyme provides for the abundant release of urea into the environment by mammals, and the latter enzyme allows the use of this urea as a nitrogen source by bacteria, fungi and plants. The recent crystal structure determination of the Ni_2^{2+} -urease from the bacterium *Klebsiella aerogenes*¹⁸ reveals some interesting parallels between these two enzymes which have evolved to catalyse urea chemistry. Both arginase and urease contain a binuclear transition metal cluster, and both enzymes contain roughly similar constellations of catalytically important carboxylate and imidazole groups in their active sites. Interestingly, Mn_2^{2+} -substituted urease exhibits catalytic activity, albeit 2% of that of the native Ni_2^{2+} -urease¹⁹; however, preliminary results with Ni_2^{2+} -substituted arginase reveal no measurable catalytic activity (D.E.A., unpublished results). Optimal catalytic activity in each system seems to have evolved with high selectivity for one particular binuclear metal cluster of specific composition and structure. Further structure-based protein-engineering experiments will allow us to probe the relationships between metal selectivity and catalysis in arginase, and

such experiments may allow us to expand upon its catalytic versatility. □

Methods

The crystallization and preliminary X-ray diffraction analysis of rat liver arginase has been reported previously¹¹. Arginase crystals diffract to 2.1 Å resolution and belong to space group $P3_1$ with hexagonal unit-cell dimensions $a = b = 88.5$ Å, $c = 106.2$ Å, with one 105K trimer in the asymmetric unit. Phase determination by multiple isomorphous replacement was hindered by chronic non-isomorphism between native and heavy-atom derivative crystals, as well as non-isomorphism among native crystals themselves (the c -axis length typically ranged from 104–115 Å)¹¹. Diffraction data were collected at room temperature on an R-Axis IIc

image plate area detector. Data reduction was performed with MOSFLM²⁰ and CCP4²¹. For phasing, initial heavy-atom positions were determined in difference Patterson maps and refined with the program PHASES²². Heavy-atom binding indicated that the noncrystallographic symmetry (NCS) axis of the trimer was tilted $\sim 9^\circ$ away from a normal to the a - b plane. The model was fit into an electron-density map calculated with solvent-flattened NCS-averaged phases at 3.0 Å resolution. Subsequent refinement and rebuilding of the native model was done with X-PLOR²³ and O²⁴, respectively. Group B factors were refined and a bulk solvent correction was applied. In the final stages of refinement, the quality of the model was improved by gradually releasing the NCS constraints into appropriately weighted restraints as judged by R_{free} . Refinement statistics are recorded in Table 1. The final protein model has excellent stereochemistry with only Gln 64 adopting a disallowed ϕ/ψ conformation. This residue is located in a type II' β -turn between strand 2 and helix B and is characterized by clear and unambiguous electron density.

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CORRESPONDENCE and requests for materials should be addressed to D.W.C. (e-mail: chris@xtal.chem.upenn.edu). Atomic coordinates have been deposited in the Brookhaven Protein Data Bank with accession code 1RLA).

ERRATUM

Transduction of bitter and sweet taste by gustducin

Gwendolyn T. Wong, Kimberley S. Gannon
& Robert F. Margolskee

Nature **381**, 796–800 (1996).

THE *Nature* cover relating to this letter, in the 27 June issue, did not do justice to the original image. The image has now been re-scanned to emphasise the significant features (right), and the new and improved version will be available on reprints of the letter. In addition, credit should have been given in the caption to Dr Luis Ruiz-Avila. The full caption reads: “Cross section of mouse tongue epithelium demonstrating expression of the α subunit of the taste-specific G protein gustducin. The centre of the image shows a foliate taste papilla containing numerous taste buds. Gustducin-positive taste receptor cells were detected by indirect immunofluorescence with a Cy3-conjugated secondary antibody. A double exposure was taken with fluorescence and differential interference contrast settings (original magnification, 250 $\times$). See Wong *et al.*, page 796 and News & Views, page 737 for the effects on taste of “knocking-out” gustducin. (Image: Luis Ruiz-Avila, Mount Sinai School of Medicine, New York, NY and CIRIT, Barcelona, Spain.)”



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